Germ cell development in the human and marmoset fetal testis and the origins of testicular germ cell tumours

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Declaration

The studies undertaken in this thesis were the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification.

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Abstract

Normal germ cell development in the human testis is crucial for subsequent fertility and reproductive health. Disruption of testis development in fetal life can result in deleterious health consequences such as testicular dysgenesis syndrome (TDS), which includes disorders, such as cryptorchidism, hypospadias, infertility and testicular germ cell tumours (TGCT). A rat model of TDS in which rats are exposed to phthalates in utero has been validated, but does result in the development of TGCT. In humans, TGCTs result from transformation of pre-neoplastic carcinoma in-situ (CIS) cells and these CIS cells are believed to arise from human fetal germ cells during their transition from gonocyte to spermatogonia, based on their morphology and protein expression profile. It has been proposed asynchronous differentiation of germ cells in the human fetal testis may predispose fetal germ cells to become CIS cells. Studying the development of these tumours in humans is difficult because of their fetal origins and prolonged duration from initiation of impaired development to invasive disease. For this reason the use of relevant animal models that can mimic normal and abnormal germ cell development may provide new insight into how TGCT develop. The Common Marmoset monkey, a New World primate exhibits many similarities to the human in terms of reproductive biology and could represent such a model.

This thesis aimed to further characterise the origins of CIS cells in the human testis by investigating the protein expression profile of CIS cells in patients with TGCT and comparing them to established markers of human fetal germ cell types using immunohistochemistry and immunofluorescence. Quantification of the various subpopulations of CIS and proliferation within these populations was performed. The thesis also investigated the Common Marmoset monkey as a potential model of normal testis and germ cell development by comparing the differentiation and proliferation profile of germ cells with those of the human during fetal and early postnatal life. During the present studies methods were successfully developed that enabled us to use testicular xenografts to recapitulate normal development of

immature testes from marmoset and human. This involved grafting pieces of testis tissue subcutaneously under the dorsal skin of immunodeficient mice and retrieving them several weeks later to investigate their development during the grafting period. Xenografts using tissue from fetal, neonatal and juvenile marmosets were performed in addition to testes from first and second trimester human fetuses. Finally the present studies aimed to use the marmoset and the xenografting approach as systems in which to examine the effects of gonadotrophin suppression and phthalate treatment on germ cell differentiation and proliferation, with particular attention to the potential for development of CIS and TGCT.

Heterogeneous phenotypes of CIS cells were identified, mostly consistent with those seen in the normal human fetal testis, however some of these CIS cells did not exhibit the same phenotype as germ cells identified in normal fetal testes. In addition it was shown that some of the proteins considered to be 'classical' markers of CIS cells, such as the pluripotent transcription factor OCT4, were not expressed in a proportion of the CIS cells. The proliferation index of CIS cells is also significantly higher in those subpopulations with the most 'undifferentiated' phenotype (i.e. $OCT4^{+}/VASA^{-}$). The present studies have generated novel data showing that the marmoset is a good model of fetal and neonatal germ cell development, with similarities to the human in terms of an asynchronous and prolonged period of differentiation and proliferation of germ cells from gonocyte to spermatogonia. This feature is also common to the human, but not a characteristic of the rodent. Fetal, neonatal and pre-pubertal germ cell development can be re-capitulated by xenografting tissue from marmoset and human testes into nude mouse hosts. Human fetal testis grafts produced testosterone and were responsive to hCG stimulation. First trimester human testis xenografts that have not developed fully formed seminiferous cords prior to grafting can complete the process of cord formation whilst grafted in host mice. In addition, germ cells in fetal human and marmoset xenografts can differentiate and proliferate in a similar manner to that seen in the intact non-grafted testis. In the intact neonatal marmoset, suppression of

gonadotrophins resulted in a 30% decrease in proliferation, however differentiation of gonocytes is not affected. In-utero treatment of neonatal marmosets with mono-n-butyl phthalate was associated with unusual 'gonocyte' clusters, however, di-n-butyl phthalate treatment of mice carrying fetal marmoset xenografts resulted in no visible effects on germ cell differentiation or proliferation and did not result in the development of CIS or TGCT.

In conclusion, this thesis has shown that there are many subpopulations of CIS cells of which many have not been previously described. These subpopulations have different characteristics, such as variable proliferation rates and this may indicate the potential for progression or invasiveness. These subpopulations have similar protein expression phenotypes to normal human fetal germ cells although the present studies have identified some CIS cells with phenotypes that are not found in the normal human testis. This thesis has demonstrated that the marmoset is a comparable model to the human in terms of asynchronous fetal germ cell development, which may predispose this species to the development of CIS/TGCT. In addition to the use of intact marmosets, these studies have also demonstrated for the first time that testis xenografting provides a comparable system for testis cord formation, germ cell differentiation and proliferation in fetal/postnatal marmosets and fetal human testis. In addition the marmoset and xenografting models have indicated that phthalates may have minor effects on testis development in the human and marmoset but do not result in CIS or TGCT. These model systems are suitable for further investigation of normal and disrupted testis development.

Publications relating to this thesis

1. **Mitchell, R. T.**, Cowan, G., Morris, K. D., Anderson, R. A., Fraser, H. M., McKenzie, K. J., Wallace, W. H., Kelnar, C. J., Saunders, P. T. and Sharpe, R. M. (2008). Germ cell differentiation in the marmoset (Callithrix jacchus) during fetal and neonatal life closely parallels that in the human. *Hum Reprod* 23, 2755-65.

2. **Mitchell, R. T.**, Saunders, P. T., Sharpe, R. M., Kelnar, C. J. and Wallace, W. H. (2009). Male fertility and strategies for fertility preservation following childhood cancer treatment. *Endocr Dev* 15, 101-34.

3. McKinnell, C., **Mitchell, R. T.**, Walker, M., Morris, K., Kelnar, C. J., Wallace, W. H. and Sharpe, R. M. (2009). Effect of fetal or neonatal exposure to monobutyl phthalate (MBP) on testicular development and function in the marmoset. *Hum Reprod*. **9**, 2244-54.

Selected oral presentations relating to this thesis

1 Scottish Paediatric Endocrine Group Annual Scientific Meeting, Dunkeld, Scotland, January 2009:

Testicular germ cell differentiation the origins of testicular germ cell tumours and fertility preservation in survivors of childhood cancer

2 Germ Cell-Soma Interactions in Gonadal Development and Germ Cell Tumours Workshop, Baeza, Spain, October 2008:

Heterogeneous expression of germ cell proteins in human testicular carcinoma in-situ and their relationship to maturational status of Sertoli cells.

3 European Society of Paediatric Endocrinology Annual Meeting, Istanbul, Turkey, September 2008:

Carcinoma in-situ and germ cell tumours in the human testis an important late consequence of DSD

4 European Society of Paediatric Endocrinology Annual Meeting, Helsinki, Finland, September 2007:

The common marmoset as a model for investigating preservation of fertility in survivors of childhood cancer

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1 5th Copenhagen Workshop on Endocrine Disrupters, Copenhagen, Denmark, May 2009:

Human and non-human primate testicular xenografts as a model for testis development and effects of endocrine disruption

2 Germ Cell-Soma Interactions in Gonadal Development and Germ Cell Tumours Workshop, Baeza, Spain, October 2008:

Heterogeneous expression of germ cell proteins in human testicular carcinoma in-situ and their relationship to maturational status of Sertoli cells

3 European Testis Workshop, Turku, Finland, May 2008:

Germ cell differentiation in the Common marmoset *(Callithrix jacchus)* monkey. A relevant animal model for human germ cell development

4 Medical Research Council Clinical Scientists in Training Meeting, London. England, February 2008

Germ cell differentiation and proliferation in the marmoset monkey *(Callithrix jacchus)*. An animal model for human germ cell development with relevance to fertility preservation in childhood cancer survivors.

5 4th Copenhagen Workshop on Endocrine Disrupters, Copenhagen, Denmark, May 2007:

Germ cell differentiation in the fetal and neonatal marmoset testis and the effect of exposure in utero to monobutyl phthalate (MBP)

Abbreviations

Abbreviation	Definition
ABC	avidin biotin complex
AGI	anogenital index
AIS	androgen insensitivity syndrome
AMH	anti-Müllerian hormone
ANOVA	analysis of variance
AP-2γ	Transcription factor activator protein-2
AR	androgen receptor
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CAIS	complete androgen insensitivity syndrome
CG	chorionic gonadotrophin
CIS	carcinoma in situ
Ck18	cytokeratin 18
CRL	crown rump length
CTA	cancer testis antigen
Cx43	connexin 43
CYP	cytochrome P450
DAB	3,3 Diaminobenzidine
DBP	Di (n-butyl) phthalate
DEHP	Di (2-ethylexyl) phthalate
DHT	dihydrotestosterone
DMRT	doublesex and mab-3-related transcription factor
DPC	days post coitum
DSD	disorders of sex development
EC	embryonal carcinoma
EG	embryonic germ cell
ES	embryonic stem cells
FGF9	fibroblast growth factor 9
FSH	follicle stimulating hormone
GBY	gonadoblastoma locus on the Y chromosome
GDNF	glial derived neurotrophic factor
GFRa1	Gdnf family receptor alpha-1
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HH3	histone H3
HPG	Hypothalamic-Pituitary-Gonadal (axis)
hpg	hypogonadal
HRP	horseradish peroxidase
HSD	hydroxysteroid dehydrogenase
INSL3	insulin-like growth factor-3

ITGCNU	intratubular germ cell neoplasia unclassified
Kg	kilogram
KLF4	Kruppel-like factor-4
L	litre
LH	Luteinising hormone
MBP	monobutyl phthalate
MEHP	mono-(2-ethylhexyl) phthalate
mg	milligram
MIS	Müllerian inhibiting substance
Mvh	mouse vasa homologue
ng	nanograms
PAIS	partial androgen insensitivity syndrome
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PGC	primordial germ cell
PGD_2	prostaglandin D_2
PGDS	prostaglandin D_2 synthase
PLAP	placental alkaline phosphatase
POR	P450 (cytochrome) oxidoreductase
RA	retinoic acid
RIA	radioimmunoassay
SCF	stem cell factor
SCO	Sertoli cell only
SEM	standard error of the mean
SF1	steroidogenic factor 1
SHBG	sex hormone binding globulin
SMA	smooth muscle actin
SOX9	*Sry*-related High Mobility Group (HMG)-box DNA binding Protein
SRY	sex determining region of the Y chromosome
SSC	spermatogonial stem cell
sTAR	steroidogenic acute regulatory protein
TBS	tris buffered saline
TDF	testis determining factor
TDS	testicular dysgenesis syndrome
tfm	testicular feminised mouse
TGCT	testicular germ cell tumour
TGFβ	transforming growth factor β
TIN	testicular intraepithelial neoplasia
TSA	Tyramide signal amplification
TSPY	testis specific protein on the Y-chromosome
VEGF	vascular endothelial growth factor
WAGR	Wilms tumour, aniridia, genito-urinary abnormalities and mental retardation
WT1	Wilms tumour-1

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1 Literature review

1.1. Introduction

Testicular germ cell tumours (TGCT) are the most common cancer of young men and comprise 90% of testicular tumours (Moller, 2005). These tumours have increased in incidence during the last 50 years in many Western countries (Richiardi et al., 2004). TGCT have been postulated to arise during fetal life in humans (Skakkebaek, 1972a), originating from the transformation of undifferentiated fetal germ cells (Rajpert-De Meyts, 2006). The exact origin and subsequent development of these pre-neoplastic germ cells is unknown. In addition there is a lack of suitable animal models of fetal and early postnatal testis development in which to investigate the origins of these tumours.

This chapter will begin with a description of normal mammalian fetal testis development, an understanding of which is crucial for establishing relevant animal models of early germ cell development and for investigation of disorders of the testis.

1.2. The development of the bipotential gonad

The initial stages of development of the gonad are common to both males and females and this results in the development of the bipotential or indifferent gonad, which is formed in the genital ridge (Wilhelm et al., 2007). The urogenital ridges are paired structures that arise on either side of the midline in the embryo and are divided into three segments. The pronephros includes the adrenal primordium, whilst the central mesopnephros is the region from which the gonad arises. Posteriorly the metanephros gives rise to the kidney (Wilhelm et al., 2007). The genital ridge is the result of a thickening of the surface coelomic epithelium adjacent to the mesonephros (Ross and Capel, 2005);(Brennan and Capel, 2004). The mesonephros houses the primordia for the mesonephric ducts of the male and the Müllerian ducts of the female (Brennan and Capel, 2004).

1.3. Precursors of somatic and germ cells in the bipotential gonad

The germ cells and somatic cells of the gonad arise from different sources. In the mouse cells arising from the surface coelomic epithelium of both sexes will eventually become the supporting Sertoli cells in the testis, whilst in the developing ovary their fate is not restricted (Karl and Capel, 1998). This occurs at 11.2-11.4dpc and this migration may be facilitated by a discontinuous basement membrane of the coelomic epithelium that has been demonstrated by laminin staining at 11.5dpc (Karl and Capel, 1998). Whilst the somatic cells arise from a location adjacent to the developing gonad, primordial germ cells (PGCs) of both sexes migrate into the developing gonad from a distant location (Ross and Capel, 2005). In the mouse, PGCs arise from cells in the proximal epiblast, adjacent to the extraembryonic ectoderm at 6-6.5dpc (days post-coitum) (Lawson and Hage, 1994). They can be identified as PGCs from 7.2dpc based on the expression of alkaline phosphatase (Ginsburg et al., 1990). These PGCs migrate through the gut mesentery into the urogenital ridge (Ross and Capel, 2005) and populate the indifferent gonad between 10-11dpc (Fig. 1.1). In the human it has been described that PGCs migrate from the yolk sac and enter an indifferent gonad at 5 weeks (Waters and Trainer, 1996). The migration of PGCs involves a combination of intrinsic factors and extrinsic signals to ensure that the cells end up in the genital ridge. In the mouse this includes the c-Kit (KIT) receptor on the surface of the germ cells and the KIT ligand from the surrounding cells, in addition to several other signalling and adhesion molecules (Richardson and Lehmann 2010).

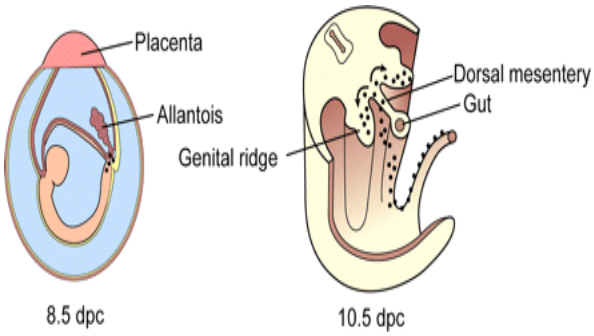

Figure 1.1. The migratory pathway of primordial germ cells. Schematic representation of the localisation of PGCs (black dots) at the base of the allantois around the hindgut pocket in an 8.5 dpc mouse embryo (*left*) and their migration along the hindgut, dorsal mesentery, and into the genital ridges in a 10.5 dpc embryo (*right*). Taken from (Wilhelm et al., 2007).

1.4. Sex determination

Mammalian sex determination is the sequence of events that results in the formation of a testis or ovary from an undifferentiated gonad (Ostrer et al., 2007). This is primarily determined by the genetic sex and the presence or absence of the Y chromosome (Wilhelm et al., 2007). A Y-linked gene locus known as the testis determining factor (TDF) houses the Sry (sex determining region of the Y chromosome) gene (Wilhelm et al., 2007);(Lovell-Badge and Robertson, 1990). *Sry* has been shown to be the gene that dictates development of the testis. This has been demonstrated by the development of a testis in XX mice with transgenic expression of Sry (Koopman et al., 1991). The converse is also true with formation of an ovary when the *Tdf/Sry* gene is mutated to an inactive form in XY mice (Lovell-Badge and Robertson, 1990);(Koopman et al., 1991);(Berta et al., 1990). Expression of *Sry* mRNA in the mouse is first detected at 10.5dpc, reaching a peak at 11.5dpc and is no longer expressed at 12.5dpc (Hacker et al., 1995). In the human *SRY* mRNA expression begins between 7-8 weeks gestation (41-44 days post ovulation) (Hanley et al., 2000).

1.5. Differentiation of pre-Sertoli cells

Differentiation of pre-Sertoli cells under the direction of SRY is a crucial step for the formation of the testis. A subset of the somatic cells that express SRY almost immediately begin to express *Sry*-related High Mobility Group (HMG)-box DNA binding Protein (*Sox9*), which is located in the long arm of human chromosome 17 (Wilhelm et al., 2007). Sox9 is also produced initially in the female gonad but its expression is not reinforced by Sry and it becomes downregulated (Kim and Capel, 2006). The formation of a testis is dependent on a critical threshold of Sertoli cell differentiation. In a normal testis, 90% of supporting cells are XY and hence express Sry as demonstrated by XX-XY chimera studies in which the ratio of XX:XY cells is in the region of 50% for somatic cells, whilst the mean ratio of XX:XY in the Sertoli cells is 10% (Palmer and Burgoyne, 1991). The fact that approximately 10% of Sertoli cells are XX indicates that they have differentiated from pre Sertoli cells without expressing Sry. Sox9 maintains its own expression in an autoregulatory loop, but

can also recruit neighbouring XX somatic cells in a paracrine fashion by upregulating prostaglandin D2 synthase (PGDS), which leads to synthesis and secretion of prostaglandin D2 (PGD2). Pgd2 acts via its receptor on the neighbouring XX cell resulting in the production of Sox9 (Wilhelm et al., 2005). Despite these 'reinforcing' mechanisms to increase Sox9 and recruit Sertoli cells, XX-XY chimeric studies in which differing proportions of XX or XY cells were mixed have shown that a threshold level of 20% of Sry expressing cells must be reached to result in the formation of a normal testis (Burgoyne and Palmer, 1993). The resultant Sertoli cells are responsible for initiating the formation of the testis cords and the differentiation of the other cell types within the testis (Wilhelm et al., 2005).

Whilst it has previously been suggested that the female pathway of differentiation is a default pathway triggered by the lack of male determining factors such as SRY and SOX9, it is now clear that there are also factors produced by the female somatic cells that 'reinforce' female sex determination. These factors include FOXL2, WNT4 and RSPO1 (Chassot et al., 2008). The Wnt signalling molecules Rspo1 and Wnt4 are expressed by XX gonadal somatic cells and can downregulate Sox9 expression (Chassot et al., 2008) (Kocer et al., 2009). An antagonistic mechanism occurs between Wnt4 and fibroblast growth factor 9 (Fgf9) to regulate Sox9 expression (Fig. 1.2). In the male Sox9 is involved in a feed forward loop with Fgf9 that results in inhibition of Wnt4, whilst in the female the absence of Sox9 allows Wnt4 to silence Fgf9 (Kim and Capel, 2006). In the absence of Fgf9 Sertoli cells do not form and the male pathway is aborted. The unopposed action of Wnt4 results in development down the ovarian pathway. This indicates that Sry is not sufficient to ensure the male pathway without Fgf9 (Kim and Capel, 2006), and indeed in XX *Wnt4*$^{-/-}$ or *Rspo1*$^{-/-}$ mice, Sox9 is upregulated despite the lack of Sry (Kim et al., 2006). Wnt4 is also required to prevent mesonephric migration of epithelial and endothelial cells that only occurs in males (Brennan and Capel, 2004);(Golestaneh et al., 2009).

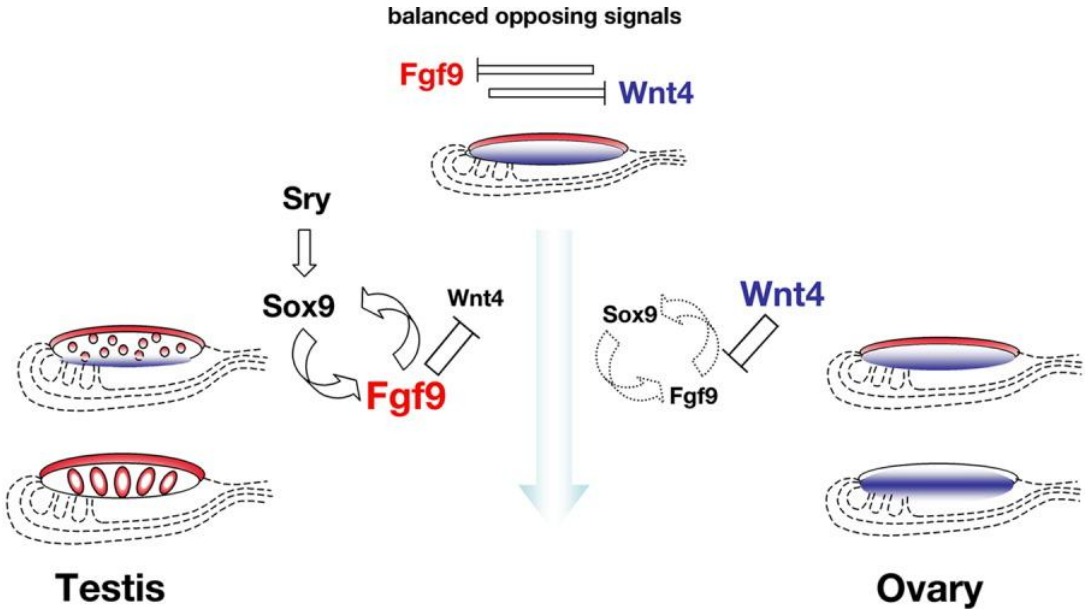

Figure 1.2. Model of balanced opposing signals between Fgf9 and Wnt4 in the development of the male and female gonad. In XY gonads, Sry upregulates Sox9 to establish a feed-forward loop that upregulates Fgf9 and silences Wnt4. In XX gonads, Wnt4 dominates and silences Fgf9 and Sox9. Taken from (Kim and Capel, 2006).

1.6. Formation of the testis cords

In the male the expression of SRY and the activation of downstream events determine the normal development of the testis. The first signs of testis differentiation can be seen at 12.5dpc in the mouse with the formation of cylindrical cords (Wilhelm et al., 2007);(Tilmann and Capel, 1999) (Fig. 1.3). This is under the control of the differentiating Sertoli cells (Tilmann and Capel, 1999) and testis cords can form even in the absence of germ cells (Wilhelm et al., 2007). In addition to its role in causing Sertoli cell differentiation, Sry is also responsible for the migration of mesonephric cells into the testis (Capel et al., 1999). Migration of cells from the mesonephros does not occur in XX gonads, but does in XY gonads (Martineau et al., 1997). Incorporation of an *Sry* transgene results in migration of cells from the mesonephros into XX gonads (Capel et al., 1999). Migrating mesonephric cells have also been shown to be able to initiate cord formation in the XX gonad using 'sandwich studies'. For example, when the XX gonad is placed between an XY

gonad and mesonephros migration of cells into the XX gonad is observed with formation of cords containing cells that expressed Sox9 (Tilmann and Capel, 1999). The peritubular myoid cells surround the cords and in collaboration with the Sertoli cells form the basal lamina that divides the testis into the cords, containing the germ cells, and the surrounding interstitium (Wilhelm et al., 2007). In the human testis, expression of SOX9 and the process of cord formation begins between 7-9 weeks of gestation, occurring over a period of some weeks (Hanley et al., 2000);(Wartenberg, 1981);(Ostrer et al., 2007).

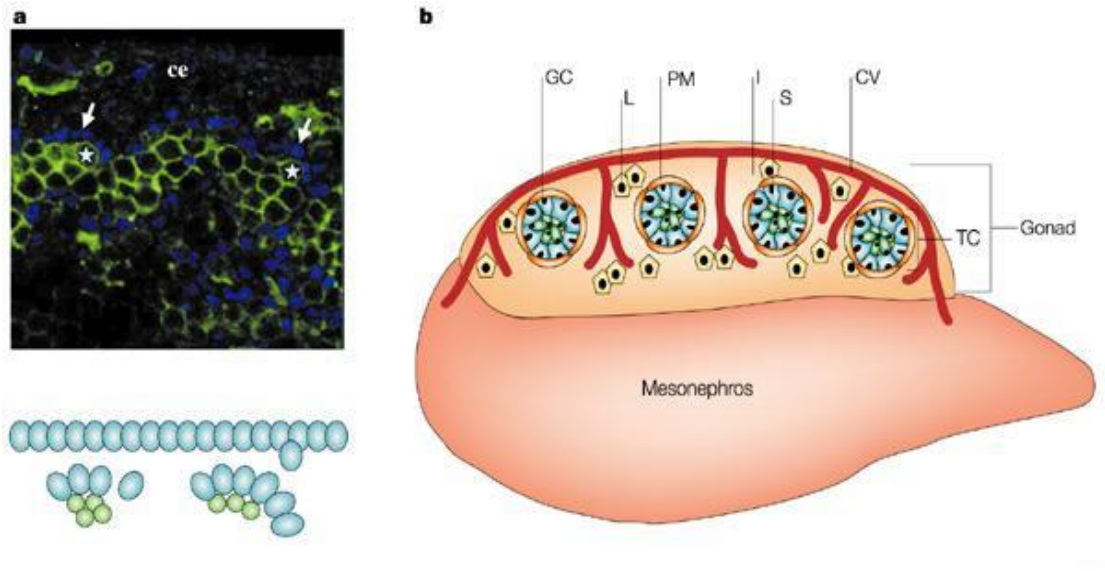

Figure 1.3. Structure of the seminiferous cords. a) At the earliest stages of testis organogenesis (11.75–12.0 days post coitum; dpc), Sertoli cells (blue) polarize and begin to aggregate around clusters of primordial germ cells (asterisk) to initiate development of testis cords. ce, coelomic epithelium. b) Between 11.5–12.5 dpc, the cells of the testis are organized into two functional compartments: testis cords (TC) and the interstitial space (I) outside the cords. Within testis cords, Sertoli cells (S; blue) surround germ cells (GC; green). A basal lamina is deposited between Sertoli cells and peritubular myoid cells (PM). The interstitial compartment contains Leydig cells (L; yellow) and the coelomic vessel (CV; red), with branches that extend between cords. Taken from (Brennan and Capel, 2004).

The relative timings of some of the key events in gonadal development in the mouse and human are summarised in Table 1.1.

	Germ cell arrival in genital ridge	*SRY* expression	Testis cord formation	Testosterone production
Mouse	9.5-11 dpc	10.5 dpc	11.5-12.5 dpc	12.5 dpc
Human	5 wks	7-8 wks	7-9 wks	7-8 wks

Table 1.1. Timing of arrival of PGCs at the genital ridge, *SRY* mRNA expression and formation of the testis cords. Comparison between the human (Wartenberg, 1981);(Hanley et al., 2000);(Ostrer et al., 2007);(Habert et al., 2001); and the mouse (Anderson et al., 2000);(Hacker et al., 1995);(Diaz-Hernandez et al., 2008);(Tilmann and Capel, 1999);(Wilhelm et al., 2007);(Habert et al., 2001). wks = weeks gestation, dpc = days post-coitum.

1.7. Development of the internal and external genitalia

The development of the gonad and the testis cords in the male is largely determined by the genetic sex and the presence of the Y chromosome as described. Once this is established then the phenotypic sex and the development of internal and external genitalia is determined (Habert et al., 2001). In the male this process is under the control of two hormones, anti-Müllerian hormone (AMH) and testosterone. AMH, also known as Müllerian-inhibiting substance (MIS), is a member of the transforming growth factor-beta (TGF-β) family of growth and differentiation factors (Behringer, 1995). It is secreted by the Sertoli cells and its expression begins shortly after SRY expression reaches its peak (Hacker et al., 1995). This glycoprotein hormone is responsible for regression of the Müllerian ducts and the absence of this hormone was shown by Alfred Jost in the 1940's to result in the formation of the fallopian tubes, uterus and upper third of the vagina (reviewed in (Rey, 2005)). Testosterone is a steroid hormone secreted by the Leydig cells of the developing testis and is responsible for masculinising the developing fetus.

1.8. Leydig cells

Leydig cells are the cells within the interstitium of the testis that are responsible for steroidogenesis and hence the production of the steroid hormone testosterone from

cholesterol (Habert et al., 2001). In addition they produce the peptide hormone insulin-like growth factor 3 (INSL3), which is responsible for the trans-abdominal phase of testicular descent in mice (Hughes and Acerini, 2008). There are at least two generations of Leydig cells. The fetal Leydig cells are responsible for masculinisation of the developing urogenital system (Svechnikov and Soder, 2008), whilst steroid synthesis by the adult Leydig cells is essential for the onset and maintenance of spermatogenesis (Habert et al., 2001).

1.8.1. Fetal Leydig cells

The origin of the fetal Leydig cell population is still uncertain although at least some of these cells migrate in from the mesonephric mesenchyme (Merchant-Larios et al., 1998), whilst others may be derived from the coelomic epithelium (Svechnikov and Soder, 2008);(Habert et al., 2001). It is also not clear whether precursor cells specific to the steroidogenic lineage exist in the bipotential gonad, or whether the lineage differentiates from multipotent somatic precursors in response to the confluence of signals from supporting cells and connective/endothelial cell lineages (Kim and Capel, 2006). Within the developing urogenital ridge there is a single population of cells that express steroidogenic factor-1 (SF1). These cells separate into two populations in regions that will become the adrenal or the gonad (Habert et al., 2001) and some of these SF1 expressing cells within the gonadal region will become the Leydig cells (Habert et al., 2001). In the mouse the fetal Leydig cells begin to produce testosterone once the cords have formed at 12.5 dpc (Merchant-Larios et al., 1993) and regress after development of the urogenital system, however they remain in the testis in adult life (Habert et al., 2001), (Gnessi et al., 2000).

In the human three phases of fetal Leydig cell development have been described (Svechnikov and Soder, 2008). Steroidogenic cells are present from 7-8 weeks of gestation (Lambrot et al., 2009) and proliferation and differentiation occurs between 7-14 weeks gestation (Svechnikov and Soder, 2008). The fetal Leydig cells are mature between 14-18 weeks, followed by an involution phase extending from 18-38

weeks (Svechnikov and Soder, 2008). As opposed to Leydig cells in the rodent, fetal Leydig cells are not present in the adult human testis. The human fetal Leydig cells begin to secrete testosterone from 8 weeks gestation, although functional differentiation with production of testosterone has been described to occur in the precursors as early as 6-7 weeks (Tapanainen et al., 1981). Between 8-10 weeks, testosterone production can probably occur in the absence of stimulation by LH or hCG, as demonstrated by the presence of Wolffian ducts (a testosterone dependent process) in a patient with an inactivating mutation of their CG/LH receptor (Kremer et al., 1995). However masculinisation of the external genitalia does not occur in these patients and this indicates that LH/CG is required for masculinisation in the human fetus later in gestation (Kremer et al., 1995). Recent studies have shown that retinoic acid can result in short term stimulation of steroidogenesis by 7 week gestation fetal Leydig cells in culture (Lambrot et al., 2006). The findings in humans are in contrast to those in male mice with mutations in the LH receptor gene, in which masculinisation of the reproductive tract during fetal life is normal and that steroidogenesis can be maintained by a number of bioactive peptides in a paracrine fashion (reviewed in (Ahtiainen et al., 2007)).

1.8.2. Adult Leydig cells

At puberty in the rat, adult Leydig cells are derived from a distinct population of undifferentiated precursors rather than the pre-existing fetal Leydig cells (Habert et al., 2001). These mesenchymal precursors proliferate during neonatal life and gradually begin to express steroidogenic enzymes and undergo morphological changes which characterise their transition from immature to adult Leydig cell (Habert et al., 2001). Once they have differentiated, the adult Leydig cells rarely proliferate. Factors that have been implicated in the differentiation and proliferation of adult Leydig cells in the rat are reviewed in (Habert et al., 2001).

In the human the fetal Leydig cells regress towards the end of the first year of life, whilst a similar population of undifferentiated mesenchymal cells as described in

the rat are located in the interstitium. At puberty these cells begin to proliferate and differentiate to adult type Leydig cells (Svechnikov and Soder, 2008).

1.8.3. Steroidogenesis

Testosterone is produced by the Leydig cells in a process known as steroidogenesis (Fig. 1.4). This process is responsible for the production of steroid hormones in the adrenal and the gonad. The production of the adrenal and sex steroids begins with a common precursor, cholesterol. A series of enzymes consisting of cytochrome P450 (CYP) and hydroxysteroid dehydrogenases (HSD) catalyse the conversion of cholesterol to the various steroid hormones (Payne and Hales, 2004). One important difference between the CYP and the HSD enzymes is that each of the CYP enzymes is a product of a single gene, whilst there are several isoforms of the HSD enzymes and they are produced by distinct genes (Payne and Hales, 2004).

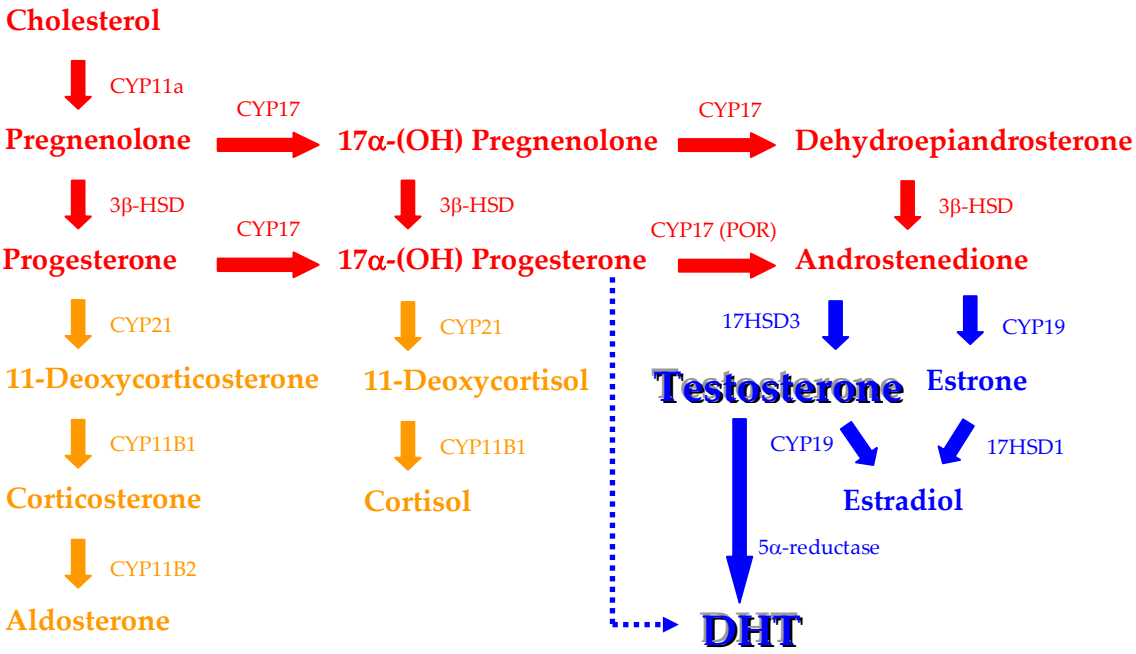

Figure 1.4. Steroidogenesis in the gonad and adrenal. Pathways in red are common to both adrenal and gonad, whilst pathways in yellow take place in the adrenal and blue in the gonad. Testosterone and dihydrotestosterone (DHT) are androgens involved in masculinastion of the male (⋯▸'back-door' pathway for DHT production). POR, P450 (cytochrome) oxidoreductase. Adapted from (Payne and Hales, 2004) and (Habert et al., 2001).

1.8.4. 3β-HSD

The 3β-HSD enzymes catalyse the conversion of pregnenolone, 17α-pregnenolone, and dehydroepiandrosterone to progesterone, 17α-hydroxyprogesterone, and androstenedione, respectively. There are several isoforms of 3β-HSD, which are expressed in a cell and tissue specific manner (Payne and Hales, 2004). Human and rat Leydig cells express 3β-HSD during fetal (Fisher et al., 2003) and postnatal life (Svechnikov and Soder, 2008), and this includes the precursor population of the adult Leydig cells (Habert et al., 2001);(Chemes et al., 1992), although expression is variable at different periods of development (Habert et al., 2001). In the human 3β-HSD is first expressed between 9-14 weeks of gestation (Gaskell et al., 2004). Once expression has begun in early fetal life 3β-HSD provides a reliable marker of the Leydig cell population throughout life.

1.8.5. Testosterone and dihydrotestosterone

Testosterone is the principal hormone produced from the Leydig cells of the testis. This hormone is converted by the enzyme 5α-reductase to the more potent androgen dihydrotestosterone (DHT). DHT rather than testsoterone is responsible for most aspects of virilisation in fetal life and also at puberty (Auchus, 2004). DHT binds more tightly to the androgen receptor (AR) and activates transcription at lower concentrations than testosterone. Although there are low levels of DHT in the testis, expression of 5α-reductase leads to local conversion of testosterone to DHT in target tissues such as the external genitalia, prostate and brain (Auchus, 2004).

1.9. Androgen receptor

In order for androgens to act within tissues the androgen needs to bind AR. The AR gene is a single copy gene that lies on the X-chromosome at Xq11–12 (Galani et al., 2008). The AR is located in the cytoplasm in the absence of ligand. Testosterone diffuses in through the cell membrane and binds to the AR, causing translocation to the nucleus and activation of transcription (Galani et al., 2008). The receptor is expressed from around 9 weeks gestation in the human testis (Gaskell et al.,

2004);(Sajjad et al., 2004). It is initially confined to the peritubular myoid cells (Murray et al., 2000);(Gaskell et al., 2004) and some interstitial cells (Sajjad et al., 2004), but is expressed in Sertoli cells in postnatal life (Chemes et al., 2008).

1.10. Chorionic gonadotrophin

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone composed of two dissimilar subunits, α and β, joined noncovalently. It is produced by trophoblast tissue in pregnancy and trophoblast disease, and also in poorly differentiated cancers. hCG exerts its effect during early embryogenesis, inducing fetal Leydig cell differentiation and testosterone production in human and primate fetal Leydig cells (Rabinovici and Jaffe, 1990). The α-subunit of hCG is similar to that of the pituitary glycoprotein hormones, whilst β-subunit is specific for hCG (Cole, 1997).

1.11. The LH/CG receptor

The human LH/CG receptor is a member of the G protein-coupled receptor family with two natural ligands, luteinizing hormone (LH) and hCG. During fetal life the human testis is exposed to high levels of hCG (Hallmark et al., 2007). The production of testosterone is dependent on placental hCG during the first trimester, whilst LH stimulation becomes increasingly important in the second trimester (Habert et al., 2001) and in postnatal life LH is responsible for testosterone production by Leydig cells, especially during puberty (Qiao et al., 2009). Importantly, exogenous hCG is able to stimulate testosterone production by postnatal human and primate Leydig cells and is used clinically both therapeutically and also for diagnostic evaluation of testosterone production in humans (Yazici et al., 2009);(Hadziselimovic et al., 2005).

1.12. The male hypothalamo-pituitary-gonadal axis

Secretion of gonadotrophins from the pituitary gland is responsible for regulating hormonal control of the gonad in the male (Fig. 1.5). The male hypothalamo-

pituitary-gonadal (HPG) axis is active from fetal life and the level of hormones produced varies at different stages throughout life. The axis regulates the onest of puberty and the establishment of spermatogenesis (Sharpe, 1994), in addition to the production of gonadal androgens. Gonadotrophin releasing hormone (GnRH) is produced by the hypothalamus and stimulates the secretion of two gonadotrophins from the anterior pituitary. These glycoprotein hormones are LH and follicle stimulating hormone (FSH). LH binds to the LH/CG receptor on the Leydig cells of the testis to promote testosterone secretion from the Leydig cells, and FSH acts on the Sertoli cells to initiate spermatogenesis. Two important negative feedback loops exist to regulate the secretion of gonadotrophins. The testosterone negative feedback loop is established in fetal life and inhibits hypothalamic and pituitary production of GnRH and LH respectively (Forest et al., 1976). Negative feedback sensitivity of the HPG axis does not develop in the rat until late in gestation (in (Hallmark et al., 2007)) as LH is not present until 16.5 dpc in the rat (Habert et al., 2001), but after birth testosterone secretion is LH dependent in rat, human and marmoset (Hallmark et al., 2007). The other negative feedback loop results from production of Inhibin-B by the Sertoli cell, which exerts inhibitory effects on FSH secretion from the pituitary gland, however this negative feedback loop is only established at around puberty (Anderson and Sharpe, 2000).

The profile of gonadotrophins and testosterone varies depending on age and development (Fig. 1.6). In the human during fetal life, the levels of testosterone are high with a peak at 14-17 weeks gestation (Reyes et al., 1974). Following birth in humans and non-human primates there is an initial rise in gonadotrophins and testosterone that continues during early infancy, the so-called 'mini puberty' (Hadziselimovic et al., 2005). In humans the rise begins at 2 weeks of life and peaks between 1 and 3 months of age (Forest et al., 1973), falling to low levels at 6-8 months. This pattern of secretion has also been demonstrated in many other primates, including the rhesus monkey and the marmoset (Dixson, 1986);(Mann and Fraser, 1996).

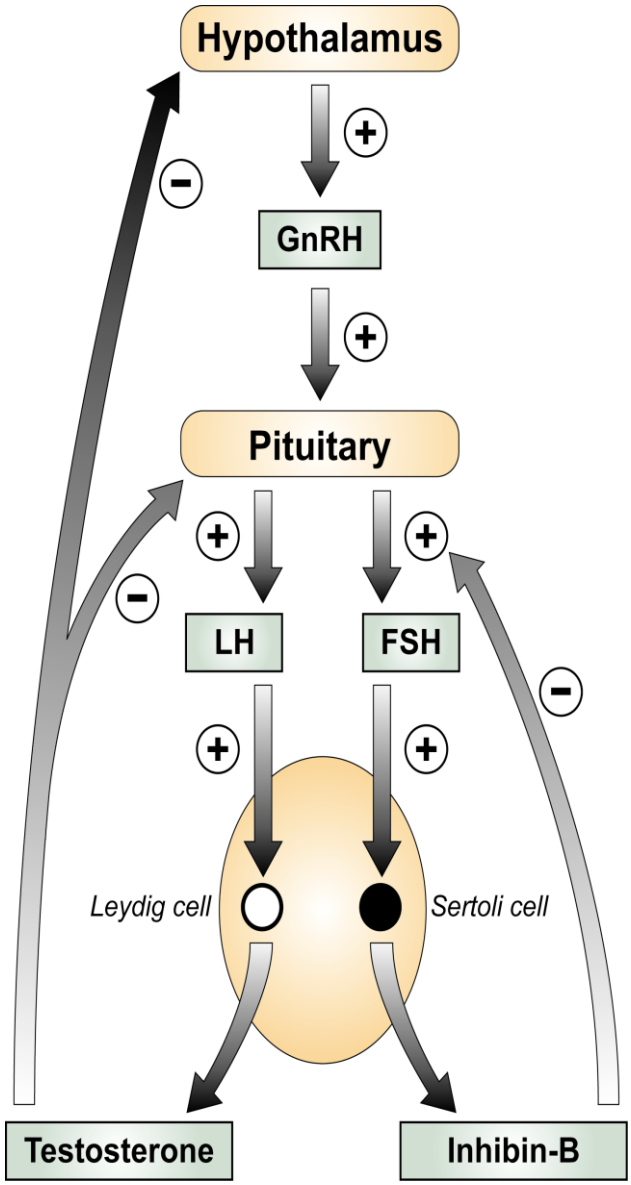

Figure 1.5. The male HPG axis. Gonadotrophins (LH and FSH) released from the anterior pituitary under the control of GnRH act on the testis to produce testosterone from the Leydig cell and Inhibin B from the Sertoli cell. Testosterone and Inhibin-B negatively feedback to the hypothalamus and/or pituitary. Stimulation (+) and inhibition (–) are indicated. Taken from (Mitchell et al., 2009).

In humans and non-human primates after the rise in gonadotrophins and testosterone during early infancy, there follows a period of relative 'quiescence' during which levels of these hormones are relatively low (Mann and Fraser, 1996). This period will be referred to as the 'childhood period', which lasts from the end of

infancy until the onset of puberty. Although this period has been described as a quiescent period it is clear that the testis is active. Sertoli cells actively express the FSH receptor, AMH and aromatase, which is the product of the CYP19 gene (Chemes, 2001). In addition there are periods of germ cell proliferation (Kelnar et al., 2002) and the transient appearance of meiotic cells (Chemes, 2001). Levels of the gonadotrophins and testosterone rise again peripubertally and remain high during adult life. In rodents there is no equivalent childhood period of low gonadotrophin and testosterone (Plant, 2006).

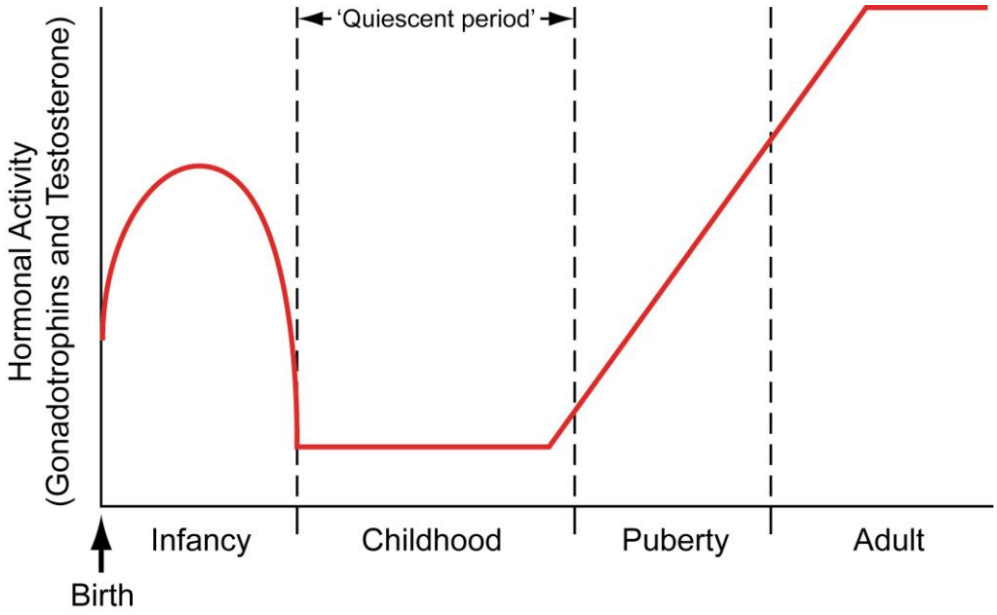

Figure 1.6. The profile of Gonadotrophin and testosterone secretion in primates. Taken from (Mitchell et al., 2009).

1.13. Disorders of sex development

In humans the term disorders of sex development (DSD) is used to describe a group of congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical (Hughes, 2008). These conditions are classified according to karyotype as either sex chromosome DSD, XY DSD or XX DSD (Hughes, 2008). Most of these disorders result from failure of normal gonadal development or the effects of abnormal androgen production, synthesis or action. One or other of the

molecules and pathways already described for gonadal development (section 1.2 – 1.8) and hormonal control of the reproductive axis (section 1.7 – 1.9) are involved in the pathogenenesis of these disorders (Hughes et al., 2006). This section will focus mainly on the 46XY causes of DSD because this thesis is concerned with the male gonad, however it must be noted that there are also many causes of XX DSD, some of which are caused by the reverse situation to those of the XY DSDs (Hughes et al., 2006). The sex chromosome DSD's include 45X (Turner syndrome), 47XXY (Klinefelter syndrome), 45X/46XY (mixed gonadal dysgenesis) and 46XX/46XY (chimeric). The latter two conditions result in ovotesticular DSD in which the gonads may contain both ovarian and testicular tissue (Hughes et al., 2006).

1.13.1. Disorders of gonadal development

During the initial stages of gonad development, lack of a functional copy of one of several genes have been shown to result in the complete absence of a gonad in mice. These include *Sf1, Wt1, Lhx1, Lhx9* and *Emx2* (Clarkson and Harley, 2002). In addition some of these genes are responsible for the development of other organs. Wilms' tumor gene-1 (WT1) is also involved in kidney formation, whilst SF1 has a role in adrenal development (Habert et al., 2001). Wt1 is expressed in the mouse gonadal ridge from 9dpc in both males and females (Pelletier et al., 1991). Wt1 has been shown to be involved in cell autonomous regulation of Sry expression (Bradford et al., 2009). Mice carrying a deletion of *Wt1* do not develop gonads and have arrested kidney development, whilst different mutations in the human *WT1* gene result in conditions that have varying degrees of renal tract anomalies and tumours, in addition to XY sex reversal (Swain and Lovell-Badge, 1999). These conditions include WAGR (Wilms tumour, aniridia, genito-urinary abnormalities and mental retardation), Denys-Drash and Frasier syndromes (Swain and Lovell-Badge, 1999). SF1 is an orphan nuclear receptor that in knockout mice results in complete absence of adrenal glands and gonads due to apoptosis in the very early stages of differentiation of these tissues (Sadovsky et al., 1995). *Sf1* knockout mice exhibit male-to-female reversal of both internal and external genital tracts (Habert et

al., 2001). A combination of adrenal failure and a female or ambiguous genitalia is also described in humans with *SF1* mutations, although depending on the mutation there may be sex reversal without adrenal insufficiency (Correa et al., 2004).

Mutations in genes involved in early testicular development, such as *SRY* and *SOX9* can result in XY sex reversal in which a genotypic male may be phenotypically female. In a study of chimeric male mice that had been constructed using XY embryonic stem cells infected in culture with MPSV.mos^{-1}neo replication defective retroviral vector, 4% of these mice displayed a female phenotype (Lovell-Badge and Robertson, 1990). These mice had ovaries and some of them were fertile. The mutations segregated exclusively to the *Tdy* (TDF in humans) region of the Y chromosome (Lovell-Badge and Robertson, 1990). Humans with *SRY*/*SOX9* mutations may present with female or ambiguous genitalia in addition to dysgenetic testis or ovotestis (Hughes, 2008). Mutations in the *SRY* gene can result in male to female sex reversal in 46XY patients and a normal *SRY* gene is absent in many 46XY subjects with a female phenotype, whereas a translocation of this gene in 46XX patients is present in most 46XX patients with a male phenotype (Habert et al., 2001). Moreover, *Sry* is the only gene needed from the Y chromosome to induce male development as shown by transgenic experiments in which XX mice carrying the *Sry* gene develop as males (Koopman et al., 1991). Studies of XX ↔ XY chimeric mice have shown that a minimum of 20% Sry expression is required for male development. Below this level an ovotestis may form, whilst levels approaching zero result in an ovary (Polanco and Koopman, 2007). Mutations in the *SOX9* gene have been linked to a severe dwarfism syndrome known as campomelic dysplasia (Foster, 1996). More than 75% of XY patients with *SOX9* mutations show sex reversal, with a gradation of genital defects (Habert et al., 2001). Conversely in 46XX patients, duplication of *SOX9* can result in female to male sex reversal (Huang et al., 1999).

Doublesex and mab-3-related transcription factors 1 and 2 (*DMRT1/2*) are genes located on chromosome 9 and are expressed in the mammalian genital ridge prior to sexual differentiation (Raymond et al., 1999). XY sex-reversal has been observed in patients with *DMRT1* mutations (Raymond et al., 1999). XY patients with mutations in the distal portion of 9p (incorporating the *DMRT* genes) have XY sex reversal associated with the loss of these genes (Ounap et al., 2004).

Whilst the genes discussed so far are mutated in XY sex reversal there are also genes that when duplicated will also result in XY sex reversal. This includes the *Dax1* and *Wnt4* genes. *Dax1* encodes an orphan nuclear hormone receptor (Brennan and Capel, 2004). In females early downregulation of Sox9 coincides with an increased expression of *Dax*1. Evidence for XY sex reversal was shown in transgenic mouse experiments, where overexpression of *Dax1* alone was able to cause XY sex reversal when tested against the weak *Sry* allele (Swain et al., 1998).

The interaction between the FGF9 and WNT4 signalling pathways as described in section 1.5, also plays a role in DSD. Male-to-female sex reversal results from mutations in *Fgf9*, whereas female-to-male sex reversal, albeit partial, occurs when *Wnt4* is mutated (Hughes, 2008). *Wnt4* is downregulated in the testis and therefore mice with a global *Wnt4* mutation resulting in a likely null allele do not have testis abnormalities, however *Wnt4* knockout in the females lack a Müllerian duct and have increased expression of steroidogenic enzymes such as 3β-HSD and 17α-hydroxylase in their ovaries (Vainio et al., 1999). This results in production of inappropriate androgens that cause female masculinisation and development of the Wolffian duct (Habert et al., 2001). An example of how several of these factors work in concert can be seen in Leydig and Sertoli cells transfected with WNT4. This leads to upregulation of DAX1, which in turn is known to antagonize SRY, hence the XY sex reversed phenotype (Jordan et al., 2001). Patients with *WNT4* duplications and XY sex reversal have also been described (Jordan et al., 2001).

1.13.2. Disorders of hormone synthesis or action

There are numerous causes of XY DSD that result from disorders of hormone synthesis or action. These can involve defects in either signalling to the Leydig cells, steroidogenesis within the Leydig cells, or impaired action of androgens once they have been produced.

Inactivating mutation of the *LHCGR* gene occurs as an autosomal recessive disorder with undervirilisation of 46 XY patients (Kremer et al., 1995). This condition is known as Leydig cell hypoplasia. The presentation ranges from complete XY sex reversal to hypospadias or micropenis, depending on the type of mutation and the degree of *LHCGR* inactivation (Qiao et al., 2009). However in approximately 50% of patients presenting with Leydig cell hypoplasia, an *LHCGR* mutation has not been found (Qiao et al., 2009).

Mutations in several of the steroidogenic or related enzymes may result in undervirilisation of the XY fetus. In these cases the gonad develops as a testis, however the external genitalia may be abnormal (Hughes et al., 2006). These defects are rare autosomal recessive conditions, which often have an adrenal phenotype in addition to the gonadal abnormalities, because of common pathways of steroidogenesis that occur within these organs (Fig. 1.4). For example, defects in the 7-dehydrocholesterol reductase DHCR7 enzyme that catalyses the final step in the synthesis of cholesterol results in the Smith-Lemli-Opitz syndrome (Szabo et al., 2009) in which a variable external genitalia phenotype is seen (Hughes et al., 2006). In contrast, defects in delivery of cholesterol from the outer to the inner mitochondrial membrane of adrenal and gonadal steroidogenic cells results in XY sex reversal and adrenal failure, a condition known as congenital lipoid adrenal hyperplasia. The protein involved in this defect is the steroidogenic acute regulatory protein (StAR) (Lin et al., 1995). Defects in enzymes that are involved in the steroid biosynthetic pathway downstream of cholesterol (Fig. 1.4), including CYP11A1, 3βHSD2, CYP17 can all result in congenital adrenal hyperplasia in addition to

phenotypic abnormalities of the external genitalia (Hughes et al., 2006). Mutations in 17βHSD3 is the most common of the disorders of androgen biosynthesis and results in a failure to convert androstenedione to testosterone (Faienza et al., 2008), however in these patients there may be partial androgenisation due to the androgenic properties of androstenedione and the external genitalia phenotype ranges from undervirilisation to complete female external genitalia (Faienza et al., 2008). Mutations in the *P450 oxidoreductase* (POR) gene results in deficiencies in the activity of a number of enzymes in the steroidogenic pathway including CYP21 and CYP17 (Hughes, 2008). The POR protein acts as a cofactor for these steroidogenic enzymes and unlike the other single enzyme defects, can result in abnormal development of the external genitalia in both sexes (Hughes, 2008).

5α–reductase deficiency results in a failure to convert testosterone to dihydrotestosterone. This results in varying degree of undervirilisation in the XY fetus, with effects on the DHT dependent tissues such as the urogenital tubercle and urogenital sinus, whilst the testosterone dependent tissues such as the Wolffian duct develop normally (Imperato-McGinley and Zhu, 2002).

Androgen insensitivity syndrome (AIS) is an X-linked disease resulting from a variety of mutations in the *AR* gene. This results in variable virilisation of 46 XY individuals with a range of phenotypes from a female appearance to masculinised genitalia and/or incompletely descended testes (Galani et al., 2008). The patients can be classified as having complete androgen insensitivity syndrome (CAIS) or partial androgen insensitivity syndromes (PAIS). CAIS usually presents at the onset of puberty when pubic hair and initiation of menses does not occur. Breast development is normal because of aromatisation of the high levels of androgen to oestrogen. PAIS presents with variable phenotypes of undervirilisation, often in the neonatal period (Galani et al., 2008).

1.13.3. DSD and risk of testicular germ cell tumour

An important aspect of DSD is that some of the conditions are associated with a significantly increased risk of the development of testicular germ cell tumours (TGCT). Individuals with DSD and gonadal dysgenesis (i.e. 45,X/46,XY), XY patients with hypovirilisation, XX patients with Y chromosome translocations are at risk of TGCT, in addition to males with undescended testis (Cools et al., 2005);(Looijenga et al., 2007b);(Carcavilla et al., 2008). Table 1.2 shows which of the DSDs are associated with TGCT and the estimated risk (Looijenga 2007a).

Risk group	Disorder	Malignancy risk (%)	Studies	Patients
High	GD[a] (+Y)[b] abdominal gonad	15–35	12	>350
	PAIS non-scrotal gonad	50	2	24
	Frasier	60	1	15
	Denys–Drash (+Y)	40	1	5
Intermediate	Turner (+Y)	12	11	43
	17β-HSD	28	2	7
	GD (+Y)[c]	Unknown	0	0
	PAIS scrotal gonad	Unknown	0	0
Low	CAIS	2	2	55
	Ovotestis DSD	3	3	426
	Turner (– Y)	1	11	557
No (?)	5α-reductase	0	1	3
	Leydig cell hypoplasia	0	2	

Table 1.2. Risk of TGCT in the various categories DSD patients. Risk is classified into high-, intermediate-, low- and no-risk groups. Taken from (Looijenga et al., 2007b).
[a] Gonadal dysgenesis (GD; including not further specified, 46XY, 46X/46XY, mixed, partial, complete). [b] GBY region positive, including the *TSPY* gene. [c] At time of diagnosis.

In patients with gonadal dysgenesis, TGCTs arise from the pre-malignant lesion known as gonadoblastoma, whilst TGCT in a non-dysgenetic testis results from a pre-malignant lesion known as carcinoma in-situ (CIS) (Cools et al., 2006c). CIS

occurs more commonly in XY DSD individuals than in those with numerical and structural abnormalities of sex chromosomes (Slowikowska-Hilczer et al., 2003).

The presence of a defined region of the Y chromosome is a prerequisite for the development of TGCT in DSD patients (Looijenga et al., 2007b). This has been determined to be the gonadoblastoma (GBY) locus and is likely to be the testis specific protein on the Y-chromosome (*TSPY*) gene (Looijenga, 2009);(Lau et al., 2000). Evidence for the requirement for Y chromosome material in the pathogenesis of TGCT has been obtained from studies in patients with Turner syndrome, where individuals with no apparent Y chromosome material are at low risk of TGCT, whilst patients with Y chromosome material from a translocation are at intermediate risk (Looijenga et al., 2007b). Conditions that result in absence of testosterone or testosterone action will not have a significantly increased risk of development of TGCT. This explains why the risk of TGCT is much lower in patients with CAIS, when compared to those with PAIS. It also explains why patients with hypogonadotrophic hypogonadism do not develop a TGCT, despite being cryptorchid (Looijenga, 2009). This may be related to the induction of germ cell apoptosis as occurs in patients with Klinefelter syndrome and CAIS (Looijenga, 2009). TGCT will be discussed later in the chapter, however before that normal germ cell development will be described in more detail.

1.14. Proteins expressed in differentiating germ cells during fetal life

1.14.1. NANOG

NANOG is a homeobox domain protein which regulates cell self renewal (Hoei-Hansen et al., 2005);(Chambers et al., 2003). It is expressed by pluripotent cells, such as embryonic stem (ES) cells, embryonic germ (EG) cells, embryonal carcinoma (EC) cells and PGCs, but its expression is not sufficient to retain pluripotency in the absence of Oct4 expression in mouse ES cells (Chambers et al., 2003). Nuclear expression of NANOG is dependent on the formation of homodimers in the human

and Oct4 has been found to interact preferentially with Nanog dimers in mouse ES cell lines (Wang et al., 2008). Expression of both NANOG and OCT4 is down-regulated during embryonic stem cell differentiation, with concomitant loss of pluripotency (Hoei-Hansen et al., 2005).

1.14.2. OCT4

OCT4 (POU5F1) is a member of the POU family of transcription factors expressed in pluripotent cells including mouse and human ES cells and is thought to be involved in PGC survival (Looijenga, 2009). Expression is downregulated during differentiation. In mouse ES cells it has been shown that altering the level of OCT4 can result in three distinct fates. Repression of OCT4 can induce loss of pluripotency and dedifferentiation to trophectoderm, whilst an increase in expression causes differentiation into primitive endoderm and mesoderm (Niwa et al., 2000).

1.14.3. AP-2γ

AP-2γ is a DNA binding transcription factor, expressed during embryogenesis and plays an important role in the development and differentiation of the neural tube, neural crest derivatives, skin, heart, and urogenital tissues in the mouse and is also overexpressed in neoplasia including breast cancer (Hoei-Hansen et al., 2004).

1.14.4. PLAP

Placental alkaline phosphatase (PLAP) is an alkaline phosphatase normally expressed in syncytiotrophoblasts (Fisken et al., 1989) and early normal (Ginsburg et al., 1990);(Honecker et al., 2004) and pre-neoplastic (Beckstead, 1983) germ cells. The function of PLAP remains uncertain, however it is expressed during migration of primordial germ cells in the developing fetus (Ginsburg et al., 1990).

1.14.5. c-KIT

The stem cell factor (SCF) receptor c-KIT (KIT) is a receptor tyrosine kinase that activates a number of signalling pathways in response to binding of its ligand SCF.

The pathway plays an important role in the survival of differentiating ES cells (Bashamboo et al., 2006). The KIT/SCF pathway is also involved in PGC migration with biosynthesis of SCF within the gonadal ridge implicated in attracting the migrating PGCs to this region (Hersmus et al., 2008) and also has a role in preventing germ cell apoptosis (Pesce et al., 1993);(Tu et al., 2007).

1.14.6. VASA

VASA is a member of the DEAD box family of RNA-binding proteins and a germ cell specific protein in both sexes (Castrillon et al., 2000). Mutation of the mouse VASA homologue (*Mvh*) gene results in male infertility and reduced fetal germ cell number (Tanaka et al., 2000).

1.14.7. MAGE-A4

The MAGE proteins are a family of chromosome X encoded cancer testis antigens (CTA). These CTAs are expressed in normal human adult testis germ cells and in different types of cancer (Gjerstorff et al., 2007). MAGE-A4 is thought to be involved in apoptosis and cell cycle progression (Gjerstorff et al., 2007).

1.14.8. NANOS-1

The NANOS proteins are a family of RNA binding proteins involved in germ cell formation, migration and differentiation. The protein forms a complex with PUMILIO-2 (Jaruzelska et al., 2003). There are three NANOS proteins identified in mice. Disruption of *Nanos-1* does not affect germ cell development, however knockout of *Nanos-2* and *Nanos-3* results in a reduction in testis weight and loss of germ cells during fetal development (Tsuda et al., 2003).

1.15. Germ cell proliferation markers

1.15.1. The cell cycle

The cell cycle is a series of events that result in division and duplication of the cell. The cycle can be divided into different stages. Interphase consists of G_1, S-phase and G_2, during which DNA synthesis occurs between the two gap phases. Between interphase there is the M phase during which mitosis occurs. Germ cells can leave G_1 and enter a resting phase known as G_0.

1.15.2. Phospho-histone H3

Phospho-histone H3 (HH3) is a protein involved in chromatin structure. It is specifically phosphorylated (at serine 10) during chromatin condensation in mitosis. Therefore antibodies to the phosphorylated form (pHH3) can provide a reliable and technically robust method for identifying mitotic activity (Colman et al., 2006).

1.15.3. Ki67

The proliferation associated nuclear protein Ki67 is important in regulation of the cell cycle. Ki67 is expressed during G_1, S, G_2 and M stages (Schluter 1993), but not in resting cells (G_0) or early G_1 (Angelopoulou et al., 2008). Ki67 has a biological half-life of 1 hour (Angelopoulou et al., 2008).

1.15.4. Proliferating cell nuclear antigen

Proliferating cell nuclear antigen (PCNA) is a nuclear protein associated with the cell cycle (Hall et al., 1990). PCNA is also required during DNA replication and nucleotide excision repair (Angelopoulou et al., 2008). It is expressed during all stages of the cell cycle with a maximum in S and G_2 phases. The transition from the growing to the quiescent state of the cell cycle does not result in a rapid degradation of the protein and the half-life is ~20 hours (Bravo and Macdonald-Bravo, 1987).

HH3 identifies cells during the short mitotic phase of the cell cycle and as a result will be expressed in a smaller proportion of cells than Ki67 and PCNA. PCNA and Ki67 are expressed through the majority of the cell cycle. PCNA has been demonstrated in a higher proportion of cells than Ki67 in studies including a study of spermatogonial proliferation in human adult testes (Steger et al., 1998). PCNA expression is identified in a larger proportion of cells than for Ki67 for several reasons. Firstly PCNA is expressed in cells throughout the cell cycle. It also has a longer half-life than Ki67 and finally it has been show that PCNA is also involved in nucleotide excision repair mechanisms (Angelopoulou et al., 2008).

1.16. Primordial germ cells

Primordial germ cells are committed to a germ cell fate in vivo (Culty, 2009), however they express markers of pluripotency such as OCT4 and NANOG (Perrett et al., 2008). Pluripotency refers to the ability of a cell to differentiate into multiple cell types depending on the signals that the cell receives and both Oct4 and Nanog are required for the maintenance of pluripotency (Loh et al., 2006). Although these cells are committed to a germ cell fate in vivo, they have been shown to form pluripotent colonies of embryonic germ (EG) cells *in vitro* (Kerr et al., 2008). In addition to pluripotency markers, PGCs also express placental alkaline phosphatase (PLAP), which has been used to trace the migration of these cells to the genital ridge (Ginsburg et al., 1990). PGC numbers increase from ~155 to 1000 between e8.5 and e10.5 in the mouse during their migration indicating that these cells are proliferating (Tam and Snow, 1981). PGC number in the human embryo prior to 6 weeks has been estimated to be between 450-1400, with a dramatic increase in numbers after they arrive in the testis (Bendsen et al., 2003).

1.17. Gonocytes

1.17.1. Differentiation of gonocytes

The PGCs arrive in the developing gonad at which point they are usually referred to as gonocytes. PGCs and gonocytes have a distinctive morphology. They are round cells with a prominent nucleus, containing one or two nucleoli and a characteristic ring of cytoplasm (Baillie, 1964). In the human male fetus gonocytes are reported to enter a period of mitotic arrest, whilst in the female the germ cells enter meiosis. Retinoic acid (RA) is thought to be responsible for this sexually dimorphic difference between male and female germ cells based on studies in the mouse (Kocer et al., 2009). Although in the mouse RA is produced in the mesonephros in both male and female gonads, somatic cells in the male testis also produce the enzyme CYP26B1, which results in degradation of RA and prevents male germ cells from entering meiosis (Bowles et al., 2006). During fetal and early postnatal life male gonocytes begin to differentiate into spermatogonia. The differentiation of germ cells from gonocyte into spermatogonia involves changes in the morphology and protein expression profile (Gaskell et al., 2004);(Wartenberg, 1981);(Holstein et al., 1987);(Fukuda et al., 1975). Up to three distinct subpopulations of cells have been described during this transition and are referred to variously as gonocytes and prospermatogonia (Wartenberg, 1981); gonocytes and prospermatogonia (Holstein et al., 1987) or gonocytes, intermediate cells and fetal spermatogonia (Fukuda et al., 1975). The prospermatogonia have also been categorised according to their proliferative activity. These have been described as M prospermatogonia (mitotically active), T1 prospermatogonia (nondividing/resting), and T2 prospermatogonia (those that had resumed mitotic activity) (Wartenberg, 1981). In these original publications these distinctions were based on morphological differences, however we have characterised the cells as gonocytes, intermediate cells and prespermatogonia based on the expression profile of various germ cell proteins (Gaskell et al., 2004) (Fig. 1.7).

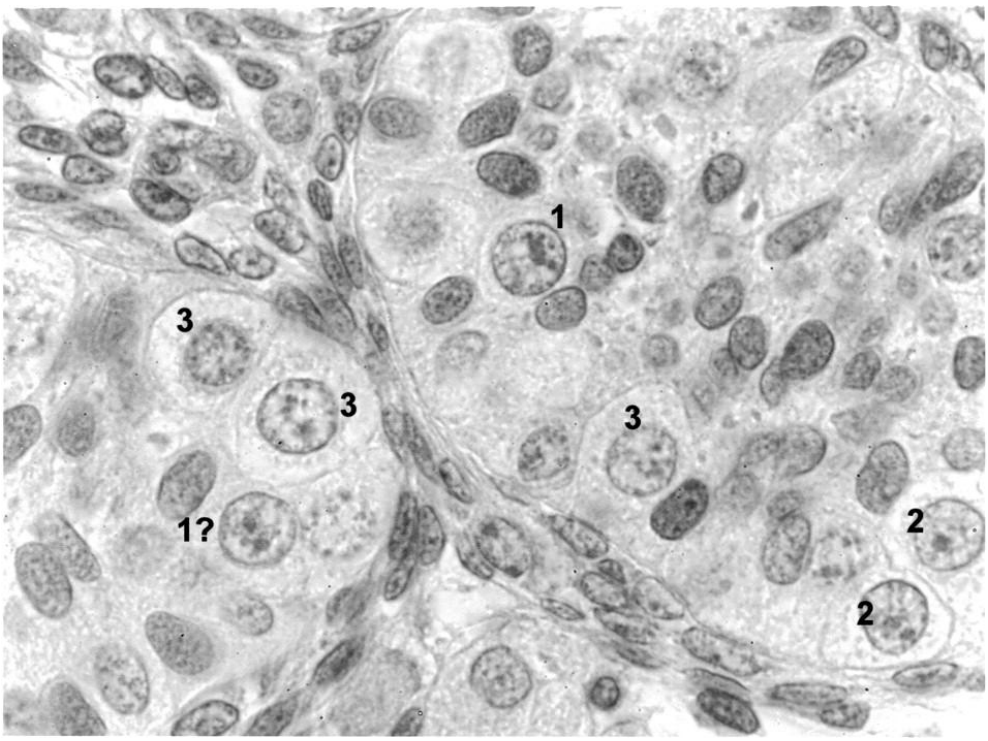

Figure 1.7. Identification of germ cells with differing morphology in second-trimester human fetal testis. Three different populations were identified. Population 1 (gonocyte): small, round cells; high nuclear:cytoplasmic ratio: prominent nucleolus: usually single cells, often in the centre of a cord. Population 2 (intermediate): round nuclei, usually in pairs, more cytoplasm/irregular outline compared with population 1. Population 3 (prespermatogonia): largest of the germ cell types, irregular outline, clear cytoplasm, usually in groups at the periphery of a cord. Taken from (Gaskell et al., 2004).

The transition between these cell types can be followed by the loss of expression of pluripotency proteins and the increasing expression of germ cell specific proteins characteristic of differentiated germ cells (Gaskell et al., 2004). Proteins associated with pluripotency (OCT4 and NANOG) and in addition proteins such as PLAP and AP-2γ are downregulated and disappear completely over the course of differentiation from gonocyte to spermatogonia (Gaskell et al., 2004);(Honecker et al., 2004);(Kerr et al., 2008);(Looijenga et al., 2003a);(Pauls et al., 2006);(Rajpert-De Meyts et al., 2004);(Jorgensen et al., 1995);(Hoei-Hansen et al., 2004);(Jorgensen et al., 1993). As expression of these proteins are downregulated, other proteins indicative of more differentiated germ cells, such as VASA and MAGE-A4 can be detected (Anderson et al., 2007);(Aubry et al., 2001);(Honecker et al., 2004);(Castrillon et al.,

2000) (summarised in Fig. 1.8). This differentiation of gonocyte to spermatogonia has been postulated to be triggered by the high levels of gonadotrophins and testosterone that occur during the neonatal period (Huff et al., 2001);(Hadziselimovic et al., 1986).

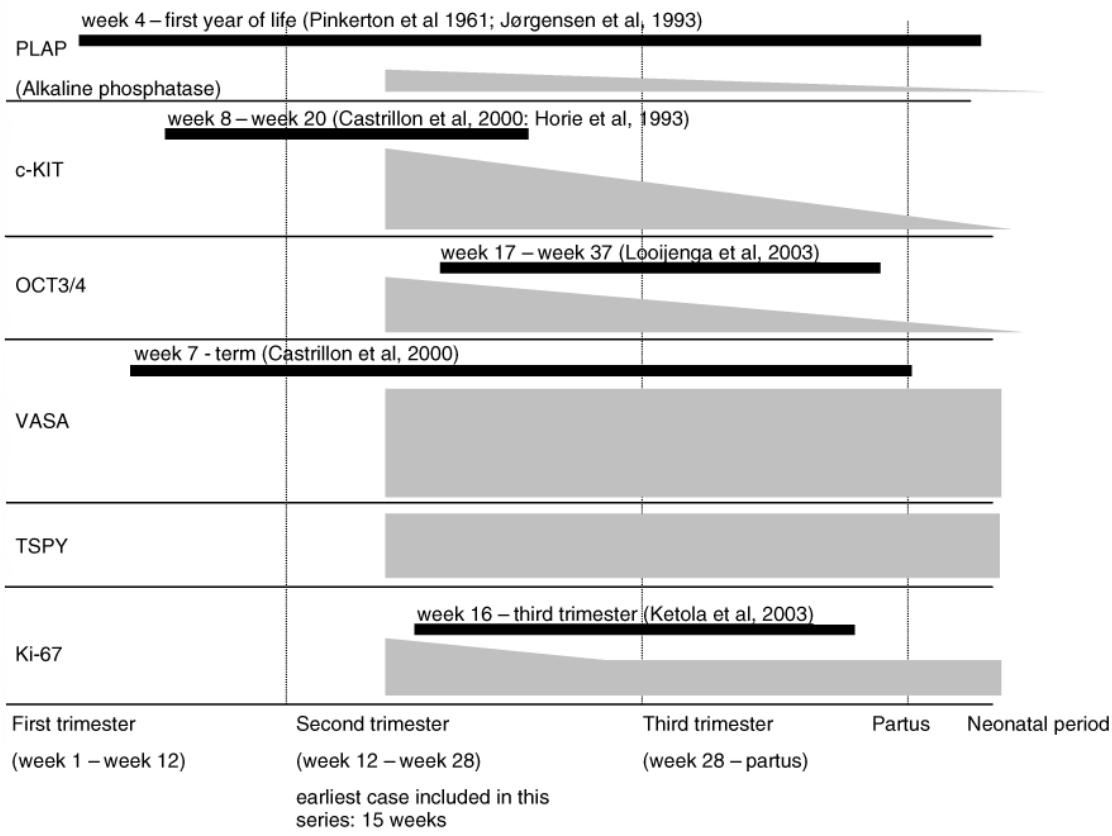

Figure 1.8. Expression of different antigens in male germ cells during intrauterine development. Findings from various studies (black bars) are compared with the findings of Honecker, 2004 (Honecker et al., 2004) (grey bars). The sizes of the grey bars schematically represent the frequency of germ cells expressing the individual factor at different developmental ages. Adapted from (Honecker et al., 2004).

Additional data relevant to this transition has also been gained by co-staining for OCT4/VASA (Anderson et al., 2007) and OCT4/MAGE-A4 (Gaskell et al., 2004). VASA mRNA and protein were low/undetectable in 1st trimester and increased in the 2nd trimester. During the 1st trimester, germ cells were OCT4 positive but did not express VASA. In the 2nd trimester germ cells with intense cytoplasmic staining

for VASA were OCT4 negative (Anderson et al., 2007). Expression of MAGE-A4, KIT and OCT4 identified three subpopulations that were described as gonocytes ($OCT4^{+}/KIT^{+}/MAGE\text{-}A4^{-}$), intermediate germ cells ($OCT4^{low/-}/KIT^{-}/MAGE\text{-}A4^{-}$), and prespermatogonia ($OCT4^{-}/KIT^{-}/MAGE\text{-}A4^{+}$). The transition was described with most germ cells demonstrating a gonocyte phenotype in the first trimester, however, from 18 weeks of gestation, prespermatogonia were the most abundant cell type. At a given age in the human fetal testis there is a mixture of germ cells at different stages of differentiation, ranging from gonocyte to spermatogonia (Anderson et al., 2007);(Gaskell et al., 2004). Occasional gonocytes can be identified in the infantile human testis based on the expression of pluripotency markers such as OCT4 (Looijenga et al., 2003a) and NANOG (Hoei-Hansen et al., 2005).

The markers described above are either expressed early and subsequently downregulated, or expressed later in development and remain present in the germ cells. However, KIT is expressed in a bimodal pattern (Culty, 2009). In the mouse, KIT is expressed by gonocytes and differentiating spermatogonia but not in the SSC population (von Schonfeldt et al., 2004). In the human KIT is also expressed in gonocytes (Robinson et al., 2001) and not in the pre-spermatogonium (Gaskell et al., 2004), but KIT mRNA (Palumbo et al., 2002) and protein are subsequently found in the adult human testis and localised to the spermatogonia (Unni et al., 2009).

There are some key differences in gonocyte differentiation in the rodent compared to the human. In mice, expression of Vasa is initiated in Oct4 positive PGCs as they enter the gonadal ridge and persists throughout the differentiation of germ cells (Anderson et al., 2007). In addition Oct4 is downregulated in a synchronous fashion, during a short time window. In the rat this occurs between e15.5 and e19.5 (Ferrara et al., 2006), whilst in the mouse this takes place between 17-20dpc (Zayed et al., 2007). Expression of gonocyte markers has ceased completely prior to birth in rodents (Ferrara et al., 2006);(Zayed et al., 2007).

1.17.2. Proliferation of gonocytes

Gonocyte numbers have been shown to increase ten-fold between 6-9 weeks post-coitum in the human fetal testis, rising from ~3000 to ~30 000 (Bendsen et al., 2003). During this period the ratio of germ cells to Sertoli cells remains constant at 1:10 (Bendsen et al., 2003). A huge increase in germ cell number takes place between the first and third trimester and the estimated number of prespermatogonia in the latter is ~4 000 000. The ratio of germ cells to Sertoli cells has been calculated at a constant level of approximately 1:6 from 10-41 weeks gestation (Waters and Trainer, 1996);(Pauls et al., 2006). In humans, it has been reported that there is only one mitotic phase in gonocytes, occurring between the third and sixth months of gestation, followed by a quiescent phase until postnatal life (Ketola et al., 2003);(Culty, 2009), whilst other reports describe a gradual cessation of premeiotic proliferation between fetal weeks 12-18 (Pelliniemi, 1993);(De Felici et al., 2004). However it has also been noted that Ki-67 staining can be detected in gonocytes throughout the second and third trimesters and into postnatal life (Honecker et al., 2004);(Berensztein et al., 2002);(Pauls et al., 2006) so further studies are required to resolve this issue. In rodents there is a fetal phase of proliferation during which a large proportion of gonocytes are proliferating. In mice this has been reported to occur between 13.5-16 dpc, (Culty, 2009) following which they are arrested in G1 (Vergouwen et al., 1991), however a recent study has described mitotic arrest occurring slightly earlier at E14.5 (Western et al., 2008). In the rat, proliferation occurs between 13-18dpc (Culty, 2009);(Ferrara et al., 2006). After this period of proliferation there is a period of quiescence until after birth when the germ cells begin proliferation again. This process occurs in a synchronous manner, similar to that described for germ cell differentiation in rodents (Vergouwen et al., 1991);(Nagano et al., 2000).

1.18. Spermatogonia

1.18.1. Differentiation of spermatogonia in the immature testis

Differentiation of gonocytes to spermatogonia can be demonstrated by morphological changes that coincide with the loss of expression of gonocyte markers. For example, in the human, PLAP expression ceases first with a minimal proportion of germ cells positive at 19 weeks gestation (Gaskell et al., 2004), although occasional PLAP positive germ cells are described in early postnatal life (Honecker et al., 2004). NANOG expression is lost in human germ cells by 3 months (Hoei-Hansen et al., 2005);(Rajpert-De Meyts, 2006), whilst OCT4 (POU5F1) is described as being present in germ cells until 3-4 months of postnatal age (Cools et al., 2005);(Rajpert-De Meyts et al., 2004). An important difference in expression of OCT4 occurs between mice and humans. OCT4 continues to be expressed in the spermatogonial stem cells of the mouse into adulthood (Pesce et al., 1998);(Ohbo et al., 2003);(Tadokoro et al., 2002), whilst OCT4 is not expressed beyond the gonocyte stage in the human (Looijenga et al., 2007b). VASA (Castrillon et al., 2000), MAGE-A4 (Aubry et al., 2001) and NANOS-1 (Jaruzelska et al., 2003) continue to be expressed in spermatogonia in humans. There are several subtypes of spermatogonia within an individual species and a variation between different species (Jahnukainen et al., 2006) (Fig. 1.9).

Spermatogonial stem cells (SSC) undergo a process of self-renewal, which maintains their numbers, whilst also giving rise to the differentiating germ cells that will ultimately enter the process of meiosis during spermatogenesis and produce spermatozoa (Ehmcke et al., 2006a). In primates the (A_{dark}) spermatogonia are thought to be the spermatogonial stem cell and to act as the regenerative reserve, while (A_{pale}) spermatogonia are the progenitor cells acting as the functional reserve (Jahnukainen et al., 2006). In the rhesus monkey the A_{pale} spermatogonia form clones and the subsequent differentiation and proliferation occurs in synchrony in these clones (Ehmcke et al., 2005). In the human the (A_{dark}) spermatogonia are thought to be found in the testis from 2-3 months of postnatal age (Huff et al., 2001). In the

mouse A-single (A_s) spermatogonia are considered to be the stem cells and it is suggested that the A_s spermatogonia act as both the stem cells and the progenitor cells (Jahnukainen et al., 2006).

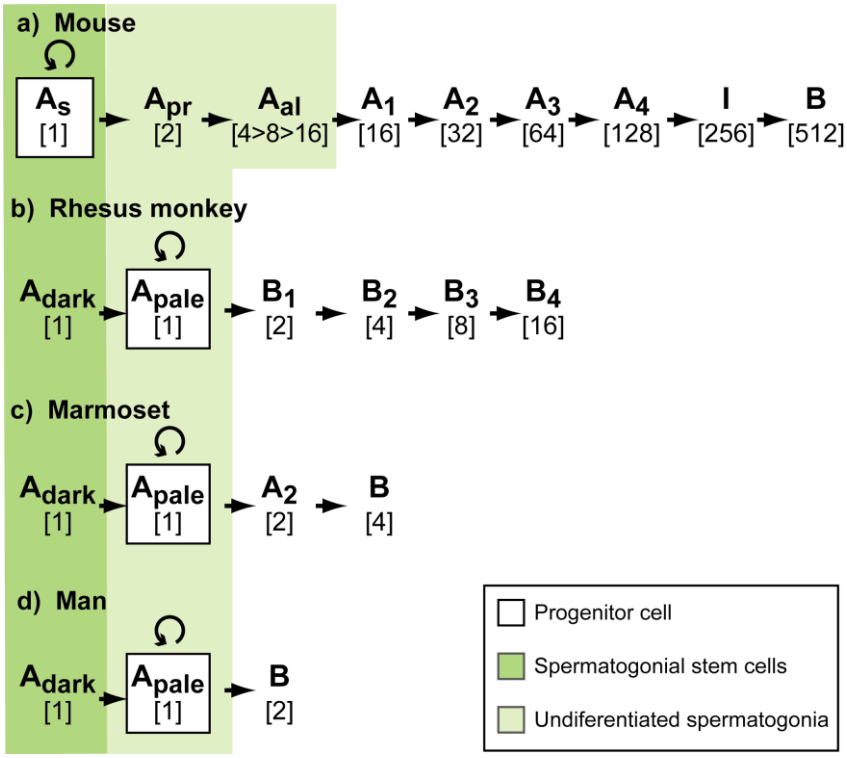

Figure 1.9. Differences in spermatogonial sub-types between species. Potential number of cells generated are shown in brackets. A_s = A-single, A_{pr} = A-paired, A_{al} = A-aligned. Taken from (Mitchell et al., 2009), adapted from (Jahnukainen et al., 2006).

Spermatogonia are the only germ cell type present within the primate testis from infancy until the beginning of puberty, apart from the transient appearance of meiotic cells that ultimately degenerate (Huff et al., 2001);(Chemes, 2001).

Spermatogonial stem cell survival is important for future generation of gametes. These SSC are thought to be located within a unique micro-environment known as the spermatogonial stem cell niche, which is formed by the supporting Sertoli cells, basement membrane, interstitial compartment and the vasculature (Hofmann, 2008). The importance of the niche has been shown in Drosophila, where division of stem

cells occurs perpendicular to the hub (Yamashita et al., 2003). The hub contains the somatic cells and can be considered the stem cell niche. The division results in two daughter cells, one of which remains in close association with the hub, whilst the other loses contact and hence loses the signals from the hub. This asymmetric division allows the distant cell to differentiate, whilst retaining a self-renewing cell in close contact with the niche (Yamashita et al., 2003). It is thought that a similar change in location results in differentiation of spermatogonial stem cells in the mammalian testis, with movement of cells away from the spermatogonial stem cell niche to a position in close association to the vasculature and interstitium (Yoshida et al., 2007). Supporting evidence has been provided by studies using transplantation of germ cells from mice into a recipient testis. It is believed that this requires migration of SSC to their niche on the basement membrane, adjacent to the interstitial compartment. Colonisation of the recipient testis following transplantation requires β1-integrin expression in the SSC as demonstrated by a lack of colonisation in β1-integrin knockout mice, whilst knocking out the gene in the Sertoli cells results in reduced SSC homing (Kanatsu-Shinohara et al., 2008).

1.18.2. Factors implicated in regulation of SSC

Glial derived neurotrophic factor (GDNF) is produced by Sertoli cells and studies in mice suggest it plays an important role in regulating self-renewal of SSC *in vivo*. For example, overexpression of Gdnf results in accumulation of undifferentiated spermatogonia but conversely, low Gdnf levels favour spermatogonial differentiation (Meng et al., 2000). The receptor for Gdnf is Gdnf family receptor alpha-1 (Gfra1), and is located on the cell membrane of A_s spermatogonia in the mouse testis (von Schonfeldt et al., 2004). In humans the protein is produced by the testis in both fetal and adult life (Davidoff et al., 2001). Isolation of Gfra1 cells from mouse testes has been achieved using magnetic beads and specific Gfra1 antibodies resulting in highly enriched A_s and A_{pr} spermatogonial populations (Hofmann et al., 2005). In addition, markers such as Oct4 and Plzf, a transcriptional repressor that regulates the epigenetic state of undifferentiated cells, are co-expressed in

undifferentiated spermatogonia (Buaas et al., 2004). These proteins are also co-expressed with Gfra1 (He et al., 2007). Plzf is also involved in suppressing SSC differentiation and loss of Plzf results in the shift from SSC self renewal to differentiation that ultimately results in loss of germ cells (Hofmann, 2008);(Maki et al., 2009). Kit is expressed in the differentiating spermatogonia (A_1 – B) (Filipponi et al., 2007), but not in A_s or A_{al}. In mice, proliferation of undifferentiated SSC is not affected by SCF, whilst differentiation of Kit expressing spermatogonia requires the stimulation of this ligand (Ohta et al., 2000). Plzf is not co-expressed with Kit and has been shown to repress Kit expression. Knockout of *Plzf* results in overexpression of Kit and this causes increased proliferation of undifferentiated spermatogonia and ultimately depletion of the SSC pool (Filipponi et al., 2007). Gfra1 positive cells isolated from mouse testes are enriched for $Oct4^+$ cells, whilst the Kit^+ population is reduced (Buageaw et al., 2005). The sorted cells demonstrated increased colony formation on transplantation into recipient testes, compared to the depleted Kit^+ fraction (Buageaw et al., 2005). Overall, these results indicate that Oct4, Plzf and Gfra1 are all expressed in SSC in mice. Kit expression is confined to differentiating spermatogonia. Despite the fact that Kit positive spermatogonia are not considered stem cells, it has been shown that Kit positive germ cells can colonize, proliferate and differentiate in recipient testes in spermatogonial transplant studies (Barroca et al., 2009). However, the efficiency is not as high as in Kit negative cells. This is postulated to occur by de-differentiation of these progenitor cells into a population of cells that have lost their Kit expression (Barroca et al., 2009), although other studies have described Kit expression in a small fraction of SSC (Shinohara et al., 2000). Of these markers for SSC, several are common to spermatogonia of rodent, non-human primate and human, such as GFRα1 and PLZF (Dym et al., 2009);(Hermann et al., 2009), whilst OCT4 is expressed in the SSC of the mouse, but not in primates (Dym et al., 2009).

1.18.3. Proliferation of spermatogonia in the immature testis

The neonatal rise in gonadotrophins and testosterone in humans is accompanied by an increase in testis weight by two-fold (Sharpe et al., 2003b) and an increase in Sertoli cell (Cortes et al., 1987) and germ cell number (Sharpe et al., 2003b) during this period. Spermatogonial proliferation, based on Ki67 expression, is relatively high in humans in the first 3 months of life, with the highest rates in the first month (Berensztein et al., 2002). It has also been reported that gonocyte proliferation does not occur in this early postnatal period (Culty, 2009). Germ cell number increases 6-fold between 0-10 years of age in humans (Muller and Skakkebaek, 1983). The A_{pale} spermatogonia have a high mitotic activity producing A_{pale} and Type B spermatogonia daughters (Ehmcke et al., 2006b). Type B spermatogonia are present from 4-5 years in the human (Culty, 2009). Spermatogonial proliferation can be increased in the immature rhesus monkey testis by treatment with testosterone or FSH (Arslan et al., 1993). There are differences between species in the theoretical numbers of germ cells that can result from each step of differentiation. More mitotic divisions of spermatogonia occur in the mouse compared to primates (Sharpe, 1994) and subsequently there is the potential for the production of many more differentiated spermatogonia (Fig. 1.9). In reality the theoretical numbers are not achieved because of apoptosis of a large proportion of the spermatogonial population (Sharpe, 1994).

1.19. Spermatogenesis

Spermatogenesis is the process by which spermatogonia proliferate and then differentiate into mature spermatozoa. This process is initiated by FSH during puberty and both FSH and testosterone appear to be required for quantitatively normal spermatogenesis (Sharpe, 1994);(Marshall et al., 2005);(Simorangkir et al., 2009);(Abel et al., 2008). Testosterone acts via the androgen receptor on the Sertoli cells, thereby exerting indirect effects on the germ cells, as evidenced by a reduced germ cell number and failure to progress beyond meiosis in mice with knockout of the androgen receptor in Sertoli cells (Abel et al., 2008). There are three phases of

spermatogenesis that are common to all mammals. The first is a phase in which the spermatogonia are undergoing frequent cell divisions and differentiating into primary spermatocytes. The final mitotic division of B-spermatogonia gives rise to diploid preleptotene spermatocytes, which undergo meiotic division to produce haploid secondary spermatocytes. These cells are located in the adluminal compartment of the seminiferous tubule. The second meiotic division results in the formation of the spermatids, which will subsequently become mature spermatozoa following the process of spermiogenesis. The seminiferous tubule is organised with the spermatogonia adjacent to the basement membrane. As the germ cells differentiate they are directed towards the lumen (Fig. 1.10).

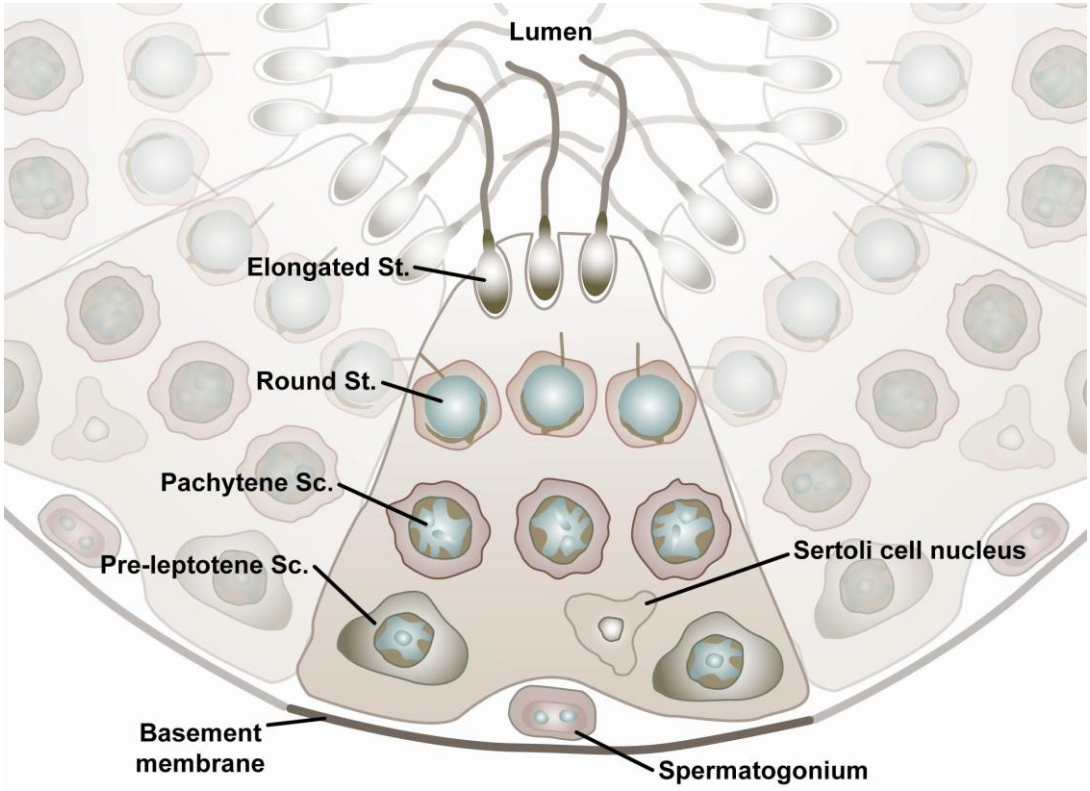

Figure 1.10. Schematic representation of a transverse view of a human seminiferous tubule. Sc – spermatocyte, St – spermatid. Taken from (Mitchell et al., 2009).

Supporting the germ cells are the Sertoli cells, which form the 'blood testis barrier' consisting of tight (occluding) junctions between adjacent Sertoli cells (Wong et al., 2004). Each Sertoli cell provides support for numerous germ cells at different stages

of development and the function of the Sertoli cells at a given stage is determined by its germ cell complement (Sharpe, 1994). The patterns of germ cell association from basement membrane to the tubule lumen are classified into stages, which are based on the morphological development of the spermatids (Weinbauer et al., 2001). Six stages have been described in the human (Clermont, 1963).

In human adults the proliferation rate of the spermatogonia has been calculated as 26%, based on Ki67 staining. This is higher in type B spermatogonia (43%) compared to the type A spermatogonia (22%) (Steger et al., 1998). Each pachytene spermatocyte gives rise to four haploid spermatids and no further mitotic division occurs once the germ cells have reached this stage (Sharpe, 1994).

Germ cell numbers can be increased by administration of FSH in adult rhesus monkeys (van Alphen et al., 1988) and this has been shown to be due to proliferation of type A_{pale} spermatogonia (van Alphen et al., 1988), (Simorangkir 2009). In these studies administration of hCG (van Alphen et al., 1988) or LH (Simorangkir et al., 2009) did not increase cell proliferation, suggesting that the threshold for a maximal impact of androgens has already been met, whilst FSH administration can augment cell function. However the importance of testosterone can be inferred from studies in the *hpg* mouse which lacks gonadotrophins and therefore has an immature testicular phenotype without full spermatogenesis, but in which replacement of testosterone induces complete spermatogenesis despite undetectable FSH levels (Singh et al., 1995). In men with low FSH levels, hCG can re-initiate spermatogenesis, although the sperm concentrations do not return to normal (Matsumoto and Bremner, 1985). This suggests that both FSH and LH are required for normal spermatogenesis although it would appear that FSH is not essential to the process as men with an inactivating mutation of the FSH receptor have varying degrees of spermatogenic impairment but are not infertile (Tapanainen et al., 1997).

1.20. Sertoli cells

1.20.1. Sertoli cell differentiation

Differentiation of Sertoli cells is associated with changes in both morphology and protein expression (Brehm et al., 2006). These changes result in fetal, pre-pubertal and adult Sertoli cell types (Fig. 1.11).

Fetal Sertoli cells (SF) have an elliptical nucleus. The nuclear morphology changes during fetal and early postnatal life to become SA-type Sertoli cells (SA). These cells have a round nucleus and are the most common type of Sertoli cell during childhood. Also present during childhood are the SB-type cells (SB). These cells have an ovoid nucleus oriented perpendicular to the basement membrane. During fetal life the Sertoli cells express proteins such as cytokeratin 18, AMH and M2A but these are downregulated at different stages during subsequent development in parallel with Sertoli cell differentiation. Cytokeratin expression ceases by 20 weeks of gestation (Rogatsch et al., 1996);(Franke et al., 2004), whilst M2A persists until at least the 5th year of life, but is downregulated before the loss of AMH (Brehm et al., 2006). AMH is first expressed at 8-9 weeks of gestation (Gaskell et al., 2004);(Franke et al., 2004) and is downregulated during puberty, reflecting terminal differentiation of Sertoli cells (Rajpert-De Meyts et al., 1998).

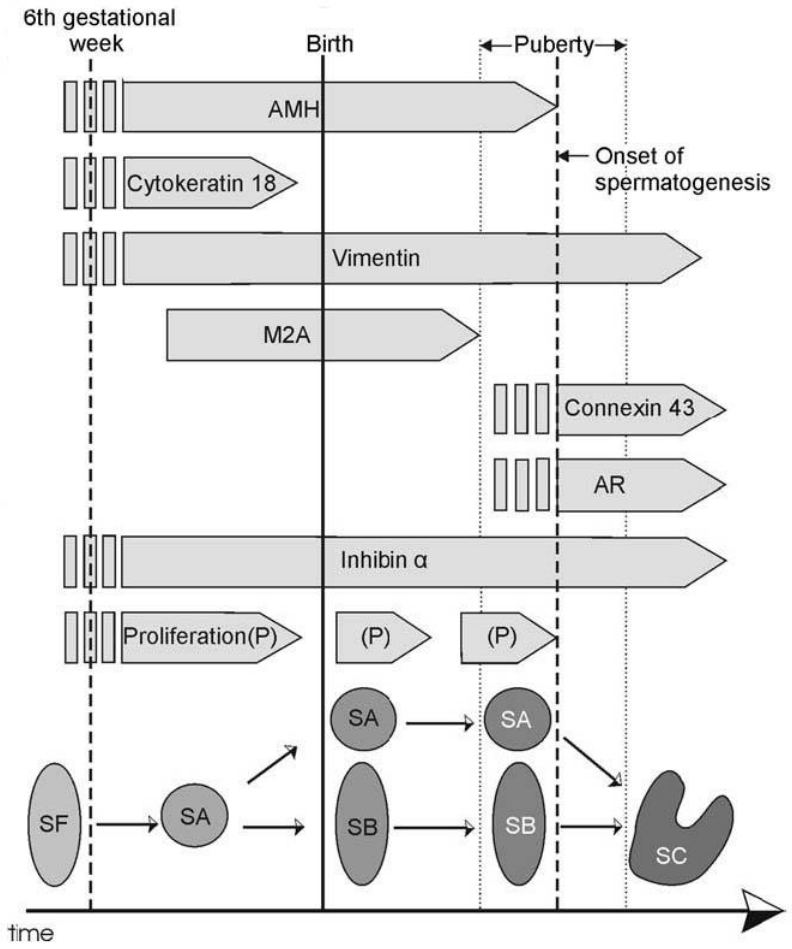

Figure 1.11. Temporal changes in Sertoli cell morphology and expression of proliferation and differentiation markers. AMH – anti-Müllerian hormone, AR – androgen receptor, P – proliferation, SF – fetal Sertoli cells, SA – SA Sertoli cells, SB – SB Sertoli cells, SC – adult Sertoli cell. Taken from (Brehm et al., 2006).

Vimentin, an intermediate filament protein, is present in Sertoli cells at all stages of development (Rogatsch et al., 1996) and as such is a stable and reliable marker of Sertoli cells throughout their differentiation (Brehm et al., 2006).

Puberty signals a change in nuclear morphology to the tripartite form with prominent nucleolus, characteristic of the adult Sertoli cell (Sharpe et al., 2003b). Protein expression changes also occur during this differentiation process. The changes in morphology and protein expression reflect the changing role of the Sertoli cell (Sharpe et al., 2003b). During fetal life the Sertoli cell is involved in initial

testis cord formation and the regression of the Müllerian duct, hence expression of AMH, whilst in adult life the Sertoli cells support spermatogenesis, which is a process that requires androgens and expression of the androgen receptor. The androgen receptor is expressed before the final maturation of Sertoli cells. In human, non-human primates and rats AR is not expressed in fetal or early neonatal life (Sharpe et al., 2003b);(Williams et al., 2001);(McKinnell et al., 2001) (Murray et al., 2000). The protein is expressed at the end of the neonatal period in marmoset monkeys, but may not be expressed until close to the onset of puberty in the human (Sharpe et al., 2003b);(Brehm et al., 2006). Another protein that is not expressed until puberty is connexin 43. This gap junctional protein is described as being necessary for the regulation of terminal differentiation of Sertoli cells and may be involved in the Sertoli cell blood testis barrier (Pelletier, 1995).

1.20.2. Sertoli cell proliferation

Sertoli cells can support a finite number of germ cells during spermatogenesis (Orth et al., 1988). This means that proliferation of Sertoli cells is an important factor in determining sperm production in adult life. Sertoli cell proliferation occurs in two phases (Sharpe et al., 2003b);(Brehm et al., 2006). In humans, primates and rats there is a period of proliferation during the fetal and neonatal period (Sharpe et al., 2003b);(Murray et al., 2000). Sertoli cell proliferation ceases during the childhood quiescent period in humans and non-human primates. Then, during pre-puberty there is a second period of proliferation. The marmoset does not exhibit a pre-pubertal period of Sertoli cell proliferation, although this does occur in marmosets treated during the neonatal period with GnRHa, allowing recovery of Sertoli cell number to normal adult levels (Sharpe et al., 2000) Once the animal reaches adulthood Sertoli cells have ceased to proliferate (Bar-Shira Maymon et al., 2003). Rats do not have an equivalent childhood period to primates and Sertoli cell proliferation continues until shortly before puberty (Sharpe et al., 2003b).

1.21. Testicular germ cell tumours

1.21.1. Germ cell tumour types

There are several types of germ cell tumour and they can occur in gonadal or extra-gonadal sites (Oosterhuis and Looijenga, 2005). Within the testis these tumours can be subdivided into three distinct groups. Each type is manifest at a different age and each has a different pathogenesis (Oosterhuis and Looijenga, 2005). Type I germ cell tumours occur in newborns and infants and are believed to originate from an early PGC/gonocyte. Type II testicular germ cell tumours (TGCT) are manifest in adolescents and young adults and arise from the pre-malignant precursor CIS cells; these tumours are divided into the seminomas and the non-seminomas. Finally there are the Type III spermatocytic seminomas that occur in elderly men (Rajpert-De Meyts, 2006) and are believed to be derived from spermatogonia (Rajpert-De Meyts et al., 2003) or spermatocytes (Oosterhuis and Looijenga, 2005).

1.21.2. Epidemiology of TGCT

At least 90% of testicular tumours are TGCT and the incidence of these tumours has been increasing worldwide over the past 60 years (McGlynn et al., 2003);(Richiardi et al., 2004). These tumours account for 60% of all malignancies in men between the ages of 20-40 (Hersmus et al., 2008). The incidence of teratomas peaks between the ages of 20-29 years, whilst seminomas peak later, usually between 30-49. The five year survival is 95% and those surviving more than five years can be considered cured (Moller, 2005).

Seminoma cells have the appearance of PGCs/gonocytes, whilst non-seminomas are capable of forming cells of all somatic, embryonic and extra-embryonic tissues (Hersmus et al., 2008), including germ cells (Honecker et al., 2006). Embryonal carcinoma is the pluripotent stem cell precursor of the non-seminomas. A summary of the development of TGCT can be seen in Fig. 1.12.

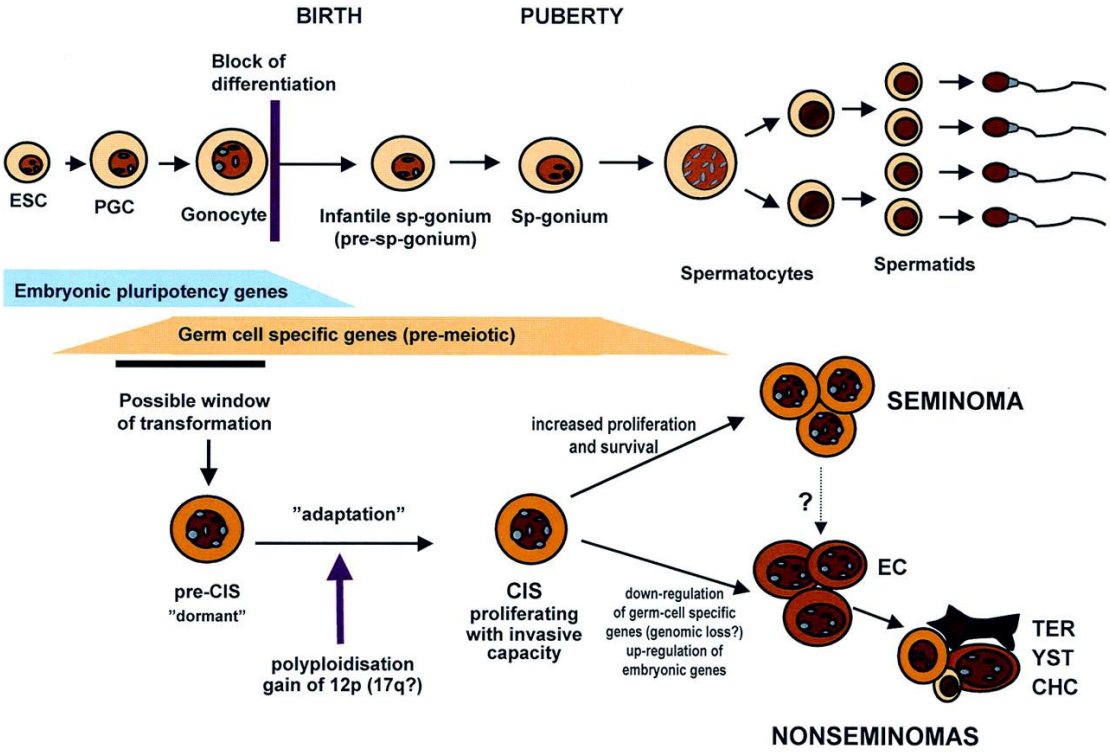

Figure 1.12. Current understanding of the pathogenesis of testicular carcinoma in situ (CIS). EC, embryonal carcinoma; TER, teratoma; YST, yolk sac tumour; CHC, choriocarcinoma. Taken from (Rajpert-De Meyts, 2006).

Two models for the generation of TGCT have been proposed. CIS cells may transform directly into either a seminoma or a non seminoma, or alternatively these CIS cells may first develop into a seminoma that may subsequently become a non-seminoma (Ulbright, 2005). Both patterns of development may also be possible (Oosterhuis and Looijenga, 2005).

1.21.3. CIS as the precursor lesion to Type II TGCT

The first description of CIS within the testis occurred in 1972 when it was noted that biopsies from the testes of two patients being investigated for infertility contained abnormal unidentified germ cells (Skakkebaek, 1972a). One of these patients subsequently developed a TGCT of the embryonal carcinoma type (Skakkebaek, 1972b). These abnormal germ cells were described as being larger in diameter than spermatogonia and contained large clumps of chromatin within the nucleus. In

addition the DNA content was noted to be higher with frequent mitotic divisions (Skakkebaek, 1972b). It was hypothesised that these cells represented a precursor lesion for TGCT and was described as carcinoma *in situ*. This hypothesis is supported by examination of their ultrastructural, histological and protein expression profiles (Skakkebaek et al., 2007);(Almstrup et al., 2005). These CIS cells are also known by several other names, including intratubular germ cell neoplasia unclassified (ITGCNU), testicular intraepithelial neoplasia (TIN) or gonocytoma *in situ* (Rajpert-De Meyts, 2006). It has been reported that all patients with CIS will eventually develop a TGCT (Oosterhuis and Looijenga, 2005). Approximately 50% of patients will develop a tumour within 5 years of diagnosis of CIS and 70% will have developed one by 7 years of follow up (Oosterhuis and Looijenga, 2005). Further evidence for CIS as the precursor lesion for TGCT is indicated by similarities in expression of germ cell proteins and chromosomal content. All invasive TGCT and CIS are aneuploid (Looijenga et al., 2003b) and most of the chromosomal imbalances detected in the invasive tumour are also present in CIS lesions (Oosterhuis and Looijenga, 2005). Gain of chromosome 12p (which contains the NANOG gene) is a consistent feature of invasive TGCT and it is postulated that this chromosome abnormality is involved in malignant progression (Looijenga et al., 2003b).

1.21.4. CIS origin from fetal gonocytes

The cell of origin for CIS has been reported to be the fetal gonocyte (Skakkebaek et al., 1987). These cells have phenotypic similarity to the gonocyte in terms of histological appearance (Skakkebaek et al., 1987) in addition to immunohistochemical expression of proteins common to both cell types such as placental alkaline phosphatase (Jorgensen et al., 1995);(Hustin et al., 1987), OCT4 (Jones et al., 2004);(Rajpert-De Meyts et al., 2004);(Looijenga et al., 2003a), NANOG (Hoei-Hansen et al., 2005), AP-2γ (Hoei-Hansen et al., 2004) and KIT (Rajpert-De Meyts, 2006) (Table 1.3). CIS cells have a large nucleus with prominent nucleoli and large amounts of glycogen granules within the cytoplasm. Other hypotheses have

been suggested for the origin of CIS cells, including spermatogonial stem cells (Clark, 2007) and spermatocytes (Chaganti and Houldsworth, 2000), however the fact that CIS can arise in pre-pubertal gonads of patients with gonadal dysgenesis (i.e. prior to meiosis) makes a spermatocyte origin unlikely and the erased imprint status of the CIS cells suggests that a PGC/gonocyte is more likely than the SSC (Sonne et al., 2008);(Looijenga, 2009)

1.21.5. Heterogeneity of CIS

Although there are many proteins expressed by CIS cells, there is some variation between individual CIS cells in their expression pattern (Rajpert-De Meyts et al., 1996) (Table 1.3). OCT4 has been reported to be present in all CIS cells (Oosterhuis and Looijenga, 2005), whilst not all CIS cells express MAGE-A4 (Aubry et al., 2001) (Rajpert-De Meyts et al., 2003). In addition, studies involving co-localisation of OCT4 and VASA have demonstrated that VASA tends to be expressed in germ cells that do not express OCT4 (i.e. normal germ cells), whilst most CIS cells express OCT4 alone. The remainder of the CIS cell population express OCT4 and VASA (Cools et al., 2005). The cause of this heterogeneity is unclear but may represent CIS cells arising from different stages during development from gonocyte to spermatogonia, and this could predetermine which kind of tumour is subsequently formed (Rajpert-De Meyts, 2006);(Almstrup et al., 2005).

Protein	PGC	Gonocyte	Sg	Sc	St	CIS	Sem	EC	Ter
NANOG	+	+	-	-	-	+	+	+	-
OCT4	+	+	-	-	-	+	+	+	-
AP-2γ	+	+	-	-	-	+	+	+	+/-
PLAP	+	+	-	-	-	+	+	+/-	-
KIT	+	+/-	+/-	-	-	+	+	-	-
VASA	+/-	+/-	+	+	+	+/-	+/-	-	-
MAGE-A4	-	+/-	+	+/-	-	+/-	+/-	-	-

Table 1.3. Expression of germ cell proteins in normal germ cells, CIS cells and TGCT. Sg – spermatogonia, Sc – spermatocyte, St – spermatid, Sem – seminoma, EC – embryonal carcinoma, Ter – teratoma. Adapted from (Rajpert-De Meyts, 2006).

1.21.6. Delay in germ cell maturation

In patients with DSD that may be at risk of TGCT it must be noted that these patients often show signs of a delay in gonocyte maturation, such that the germ cells continue to express gonocyte markers such as OCT4 and PLAP beyond infancy (Cools et al., 2005). This can make diagnosis of CIS in these children difficult. However there are suggested criteria to differentiate between these conditions. These include the age of the patient, position of OCT4 cells within the tubule and their distribution throughout the gonad. Younger age, luminal position and widespread distribution indicates maturation delay, whilst older children with OCT4 cells on the basal lamina and located in a specific region of the testis is suggestive of CIS (Cools et al., 2006a). Recently it has been reported that the stem cell factor (SCF) is expressed in CIS cells, but is negative in fetal germ cells. This is the first description of a marker that can accurately distinguish between the two and may have a role in diagnosing CIS in pre-pubertal patients with DSD and distinguishing these cells from germ cells with delayed maturation (Stoop et al., 2008).

1.21.7. Proliferation of CIS cells

During childhood, CIS cells divide at a slow rate. Around puberty these cells begin to replicate more rapidly (Brehm and Steger, 2005). The persistent expression of genes such as OCT4, are believed to be involved in the expansive proliferation of CIS cells (Cools et al., 2006b);(Rajpert-De Meyts, 2006). Proliferation of CIS cells results in an increase in diameter of the tubule, which is followed by invasion of the surrounding tissue (Donner et al., 2004). Mitotic frequency has been quantified to be 0.65% in CIS, compared to 3.59% in seminoma (Hofken and Lauke, 1996). Once the CIS cells have developed into an invasive tumour they are highly proliferative. Studies of seminoma and embryonal carcinoma describe Ki67 staining in 70% and 86% of cells respectively (Datta et al., 2000). The tumour suppressor gene PTEN is expressed in normal germ cells and Sertoli cells. It acts as an antiproliferative and pro-apoptotic factor through $p27^{Kip}$ (Di Vizio et al., 2005). PTEN is also expressed in CIS but is lost on transformation to invasive tumour, which may reflect increased proliferation in these cells on progression. Another factor that has been associated with differentiation and proliferation of CIS cells is Kruppel-like factor 4 (KLF4). This transcription factor is expressed at very low levels in human fetal gonocytes, but is expressed at high levels in CIS, intratubular seminoma and early seminoma. This suggests that it may also be involved in promotion of proliferation and pluripotency (Godmann et al., 2008). TSPY is also expressed in CIS cells and functions in mitosis. It has been hypothesised to abolish checkpoints in the cell cycle and result in increased growth and proliferation (Lau et al., 2009). Cx43, expressed by the Sertoli cells, has also been implicated in the control of proliferation of CIS cells. Cx43 expressed by the Sertoli cells induces p21 (Brehm and Steger, 2005), which is a cyclin-dependent kinase implicated in the control of the Gl to S phase transition in mammals (Harper et al., 1993). This induction of p21 reduces germ cell proliferation and may help to maintain the long latency period of CIS during childhood (Brehm and Steger, 2005). As the intercellular communication between Sertoli cell and CIS cell breaks down the CIS cells may begin uninhibited proliferation (Brehm and Steger, 2005).

1.21.8. The relationship between Sertoli cell differentiation and testis pathology including CIS

A close relationship exists between the Sertoli cell and the germ cells within the testis. Therefore there has been interest in the relationship between the Sertoli cells and the associated CIS cells in the testes of patients with TGCT. Cytokeratin 18 (Ck18) is expressed by the Sertoli cells of the human fetal testis and downregulated postnatally and is not expressed in the normal adult testis (Brehm et al., 2006) (Stosiek et al., 1990). Expression of Ck18 has been described in adult patients with CIS (Stosiek et al., 1990);(Brehm and Steger, 2005), in addition to other testicular disorders such as SCO syndrome and spermatogenic arrest at the level of spermatogonia (Steger et al., 1996);(Maymon et al., 2002). It is reported that tubules with CIS as the only germ cell type express CK18, whilst tubules containing CIS and normal germ cells (including spermatocytes) may be either positive or negative. Tubules that contain CIS along with round or elongate spermatids are negative for Ck18 (Kliesch et al., 1998). This could reflect de-differentiation of Sertoli cells in some of the tubules resulting in the re-emergence of an immature phenotype in these tubules (Brehm and Steger, 2005). Alternatively this may reflect a primary failure of maturation. Tubules containing Ck18 in patients with various abnormalities of spermatogenesis also express M2A and the loss of M2A has been described as an irreversible marker of transition of prepubertal Sertoli cells (Steger et al., 1999). M2A is not found in the adult testis in either normal situations or spermatogenic arrest (Steger et al., 1999). AMH is expressed in Sertoli cells from 7-9 weeks of gestation until puberty (Brehm et al., 2006);(Ostrer et al., 2007). The downregulation in expression of AMH coincides with the onset of androgen receptor expression, which can be detected in Sertoli cells from around the time of puberty (Brehm and Steger, 2005). In patients with AIS there is prolonged expression of AMH, which suggests that there is a link between AR dependent changes in Sertoli function and reduced expression of AMH (Rajpert-De Meyts, 1999). This is not the case in rodents. In *Tfm* mice that lack the androgen receptor, AMH is still downregulated, albeit later than in normal mice (Al-Attar et al., 1997).

In addition AMH is downregulated normally in rats with knockout of the *AR* (Tan et al., 2005). In adult patients with spermatogenic arrest there is variable expression of AMH (Steger et al., 1999), whilst in those with Sertoli cell nodules and SCO tubules AMH is often present (Brehm et al., 2006). In patients with CIS there is no expression of AMH in either normal tubules or CIS containing tubules. Androgen receptor expression is present in the Sertoli cells of normal adult testes (Brehm et al., 2006) and in CIS containing tubules (Rajpert-De Meyts and Skakkebaek, 1992).

1.22. The testicular dysgenesis syndrome

The testicular dysgenesis syndrome (TDS) is a collection of disorders that include testicular cancer, undescended testis, hypospadias/intersex (DSD) and male fertility problems with varying penetration (Skakkebaek et al., 2007). All these conditions are thought to be related to suboptimal testicular development during fetal life, which is consistent with the finding of increased rates of TGCT in patients with some forms of DSD (Looijenga et al., 2007b). The hypothesis proposes that abnormal testis development (dysgenesis), which could have numerous primary causes, leads secondarily to hormonal or other malfunctions of the Leydig and/or Sertoli cells during male sexual differentiation, leading in turn to increased risk of germ cell effects and reproductive disorders (Sharpe and Skakkebaek, 2008). Figure 1.13 summarizes the relationship between the various factors that could contribute to development of this syndrome.

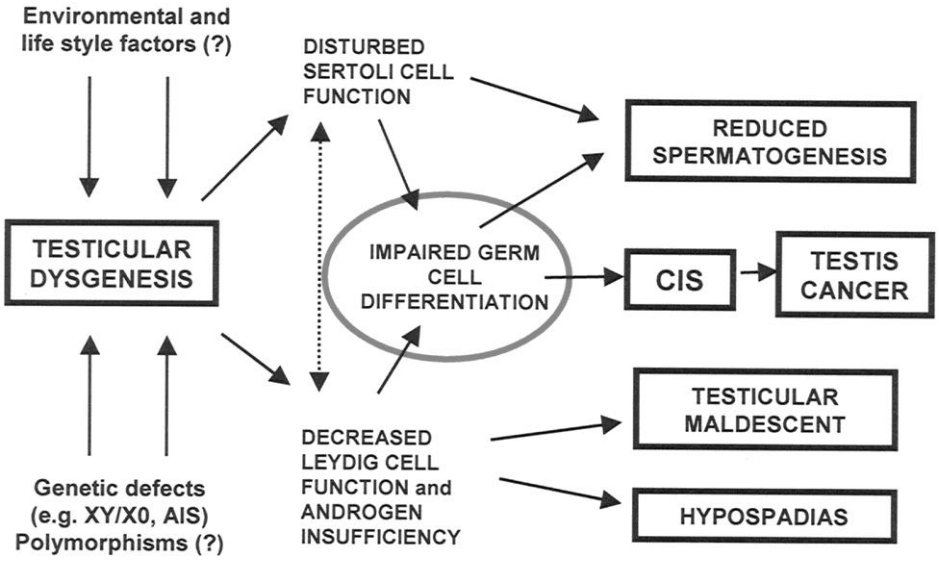

Figure 1.13. Schematic representation of the possible aetiology, pathogenesis and clinical manifestations of testicular dysgenesis syndrome. Emphasis on the key role of disturbed germ cell differentiation in the pathogenesis of testicular carcinoma *in situ* (CIS). Taken from (Rajpert-De Meyts, 2006).

Epidemiological evidence supports the existence of a TDS. Countries with the highest incidence of testicular tumours also have the highest frequencies of other male reproductive problems and this has been demonstrated clearly in the Scandinavian countries (Skakkebaek et al., 2007). In addition, investigation of patients with one of the features of TDS, revealed the presence of dysgenetic areas in their testicular biopsies (Skakkebaek et al., 2003). In patients with a TGCT, 8.7% will have CIS and 25% of patients will have other evidence of dysgenesis in the contralateral testis (Hoei-Hansen et al., 2003). Patients with TGCT have poor spermatogenesis in their contralateral testis independent of the direct tumour effects and this may be due to pre-existing dysgenesis in the contralateral testis (Dieckmann et al., 2007). Also, men with infertility are 2.8 times more likely to develop TGCT (Walsh et al., 2009) and patients with TGCT have fathered significantly less children prior to diagnosis than men without TGCT (Norgil Damgaard et al., 2002).

The rapidity of the increase in TGCT over time suggests that TDS has an environmental component (Skakkebaek et al., 2001) and this has been supported by Scandinavian migration studies, which show that TGCT risk in the second generation of migrants becomes equivalent to the local population (Skakkebaek et al., 2007). Oestrogens, anti-androgens and other endocrine disruptors such as phthalates have all been suggested as potentially involved in the development of these disorders (Skakkebaek et al., 2001).

1.22.1. Animal models of TDS

An animal model of TDS has been described in rats treated with phthalates in utero. Phthalates (phthalic acid esters) are plasticizers found in most plastics and in many types of packaging and common household items such as soap, shampoo, cosmetics and hairspray (Rajpert-De Meyts et al., 1996). They form part of the polymer network but are not chemically coupled to it and therefore they are able to leach out of these products (Lucas et al., 2009). They are one of a heterogeneous group of chemicals known as endocrine disruptors. Treatment of rats during pregnancy with Di(n-butyl) phthalate (DBP) results in offspring with high rates of hypospadias, cryptorchidism and testicular atrophy in a dose dependent manner (Mylchreest et al., 1998). These effects can be achieved by treating between gestation day 12-21 (Mylchreest et al., 2000). It was noted that these were similar effects that were seen with anti-androgens (Mylchreest et al., 1998). Whilst these studies demonstrated gross pathology of the reproductive tract, further studies identified histological and histochemical features that were in keeping with other features of the proposed human TDS, including dysgenetic seminiferous cords, SCO tubules, tubules with incomplete spermatogenesis, Sertoli cell immaturity, intratubular Leydig cells (Fisher et al., 2003) and Leydig cell aggregation (Mahood et al., 2005). These features were noted in addition to those described previously such as hypospadias, cryptorchidism and infertility. In addition, immaturity of Sertoli cells as described in the DBP study is also seen in patients with CIS, SCO and infertility (Stosiek et al., 1990);(Brehm and Steger, 2005);(Steger et al., 1996);(Maymon et al., 2002). These are

also part of TDS in humans. The discovery of intratubular Leydig cells has also been described in patients with infertility and cryptorchidism (Mori et al., 1987), further supporting the validity of the DBP treated rat as a model of TDS. The areas of dysgenesis in these studies were described as being similar to areas of testicular dysgenesis found in the testes of patients with TGCT (Hoei-Hansen et al., 2003). However, although Oct4 expression persisted at e17.5 in a higher proportion of gonocytes in DBP treated animals compared to controls, it was no longer expressed in any of the germ cells at e19.5 and importantly there was no evidence of CIS or TGCT in the treated animals (Ferrara et al., 2006).

Some of the effects of DBP in fetal rats can be explained by androgen suppression. There was a 90% decrease in intratesticular testosterone in DBP treated animals at gestational day 19, compared to controls and this could explain the hypospadias as inhibition of normal male external genitalia development can be induced by treatment with the anti-androgen flutamide (Imperato-McGinley et al., 1985). However the abdominal position of the cryptorchid testis is less likely to be explained by impaired androgen action. Although androgens are responsible for the latter stages of testis descent, abdominal descent of the testis is mediated by Insl3 in mice, as evidenced by the bilateral cryptorchidism that results from targeted deletion of the INSL3 gene (Zimmermann et al., 1999). Mice double mutant for *AR* and *Insl3* have testes positioned adjacent to the kidneys, steadied by the cranial suspensory ligament, rather than free moving within the abdomen as seen in the INSL3 mutant (Zimmermann et al., 1999). This demonstrates that androgens play a role in release of the testis for descent, however in conditions with impaired androgen signalling in the human, such as CAIS, abdominal testis descent still occurs (Hughes and Acerini, 2008). Even if androgen suppression did play a role in the cryptorchidism, it does not play a role in the development of multinucleated gonocytes and Leydig cell aggregates. (Scott et al., 2008). Immaturity of Sertoli cell maturation in DBP treated rats was also described as being independent of androgen suppression, as fetal Sertoli cells of treated rats do not express the AR

(Sharpe et al., 2003b). However a more recent study has reported that the effects on Sertoli cell maturation occur in adulthood as a result of dedifferentiation of Sertoli cells in the absence of germ cells (Hutchison et al., 2008).

1.22.2. Effects of phthalates in other species

Although phthalates have been shown to cause a TDS-like syndrome in rats the effects of these chemicals in other species is variable. In particular the effect on androgen production that occurs in rats does not occur in mice (Gaido et al., 2007), although effects of phthalates are still seen in mice treated with DBP. These mice exhibit an increased number of multinucleated gonocytes per cord compared with controls, without a measurable decrease in testicular testosterone (Gaido et al., 2007). *In vitro* culture of human fetal testis with MEHP (a metabolite of DEHP) resulted in a decrease in germ cell number despite having no effect on testosterone production (Lambrot et al., 2009). A lack of effect on testosterone production has also been shown with *in vitro* cultures of human fetal testes cultured with MBP (Hallmark et al., 2007). Despite these results there is epidemiological data to suggest that male reproductive disorders are correlated with levels of exposure to phthalates. This includes a study of phthalates in breast milk and the levels of reproductive hormones in the infant. Levels of MBP were inversely correlated with serum testosterone at 3 months of age, and positively correlated with LH:Testosterone and sex hormone binding globulin (SHBG) (Main et al., 2006). Anogenital index (AGI) in offspring has also been shown to be inversely correlated with the prenatal maternal urinary MBP level (Swan et al., 2005).

1.22.3. Animal models of CIS and TGCT

Despite the presence of animal models that reflect various aspects of TDS, to date there has been no convincing animal model of TGCT. Although many types of testicular tumour are described in animal models, there are fundamental differences between them and the human TGCTs. Testicular tumours can be induced in a mouse model by over-expressing *Gdnf* (Meng et al., 2000) and these tumours

demonstrate some of the features of classical seminoma (such as expression of alkaline phosphatase), however there remain significant differences to human seminoma (Sariola and Meng, 2003). The histological appearance differs between the two and areas of normal spermatogenesis and lymphocytic infiltration are seen in the testes of humans with TGCT but not in this mouse model. Most importantly the precursor lesion is different between the two species. Human CIS cells and the mouse cluster cells have different morphology and distribution within a tubule. Similar clusters of cells have been described in mice expressing the HERV-K *rec* retroviral gene. These are arranged in layers and do not occur until 19 months of age (Galli et al., 2005), in contrast to CIS in humans, which develops in fetal life and consists of a ring of cells along the basement membrane. Spontaneous testicular tumours are also known to arise in the *129* strain of mice but once again these tumours are more representative of the Type 1 TGCT and do not have a pre-invasive CIS equivalent (Anderson et al., 2009);(Walt et al., 1993). Mice have also been shown to develop testis tumours after diethylstilboestrol treatment, however these are not germ cell tumours (Newbold et al., 1987).

'CIS-like' cells have been reported in rabbits (Veeramachaneni, 2008);(Higuchi et al., 2003) and canines (Grieco et al., 2008). A rabbit has been described with a small testis and a low sperm count. Histologically this animal had tubules containing normal differentiating germ cells in addition to cells with the appearance of CIS (Veeramachaneni and Vandewoude, 1999). Cells with the appearance of CIS have also been described in the testes of animals treated with the anti-androgenic pesticide DDT during fetal and neonatal life (Veeramachaneni, 2008). 'CIS-like' cells have also been described in two rabbits after treatment with DBP (Higuchi et al., 2003), however whilst one of the rabbits was treated in utero, the other rabbit was treated in adolescence. This does not fit with the concept that CIS cells have a fetal origin and casts doubt on whether these are truly 'CIS' cells. In addition, none of these animals develop tumours and as a result these animals cannot be considered a model of CIS or TGCT (Veeramachaneni, 2008).

It has been reported that 5-15% of all tumours in dogs are testicular (Ruttinger et al., 2008). 'CIS-like' cells in association with other TDS lesions have also been described in 8/32 dogs receiving orchiectomy for a variety of indications (Grieco et al., 2008), This included impaired spermatogenesis, SCO, tubular atrophy and Leydig cell hyperplasia (Grieco et al., 2008). However the CIS lesions and testicular tumours were found in aged dogs and it has been suggested that these canine seminomas more likely represent the spermatocytic seminomas and that in fact they also lack true CIS (Oosterhuis and Looijenga, 2005).

Attempts to produce an in vivo model of TGCT has also been attempted using intratubular transplantation of tumour cells into nude mice. The JKT seminoma cells are a cell line that are thought to be representative of seminoma cells. Following transplantation these cells demonstrated growth and proliferation and expressed PLAP but they did not express OCT4/TSPY or KIT (Li et al., 2008). However there is some doubt as to whether the JKT cells actually represent seminoma cells (Eckert et al., 2008). The other problem with this approach is that it will not replicate the development of CIS as these cells are already transformed.

1.23. The Common Marmoset monkey

The Common Marmoset (*Callithrix jacchus*) is a New World primate (Fig. 1.14), commonly used in scientific research. It's value as a laboratory animal is enhanced by its small body size, early age of sexual maturity and relatively high reproductive rate (Holt and Moore, 1984). They are one of the least expensive primates to maintain and do not harbor pathogens of concern to human health (Abbott et al., 2003). In particular there are a number of features about the marmoset that make this primate a suitable animal model for reproductive research.

Figure 1.14. The Common Marmoset monkey.

1.23.1. Reproduction in the common marmoset

The species exhibits high reproductive efficiency with an average litter size of 2.64 (Nubbemeyer et al., 1997). The average gestation is 144 days and adult females have a median interbirth interval of 154 days (Windle et al., 1999) and can therefore produce two sets of offspring per year. This allows for an efficient production of numerous offspring over a relatively short period. Multiple births are common in the marmoset and it is thought that they most commonly produce twins in the wild, although triplet births may occur at higher frequency in captive bred animals (Windle et al., 1999). Twins produced are dizygotic but share a chorionic cavity and placental circulation which makes co-twins chimeras, including germ-line chimerism (Ross et al., 2007). The frequent occurrence of co-twins is ideal for studies that investigate a treatment effect where one co-twin is treated and the other acts as a control. Co-twins are highly comparable and should therefore minimise the number of animals required for scientific studies (Sharpe et al., 2003a).

1.23.2. Marmoset embryology and fetal gonad development

Relatively little is known about the development of the fetal testis in the marmoset. The fetal phase of gestation is relatively late in comparison with other primates and humans (Phillips, 1976). The embryonic phase lasts for 12/20 weeks in the marmoset, compared with 7-8/40 weeks in the human (Phillips, 1976). Gestation can be estimated from crown-rump length (CRL) (Table 1.4). Fetal sex can be determined histologically from a CRL of 17mm with the appearance of testicular cords (Hampton, 1971).

CRL (cm)	0.22	0.60	1.05	2.16	3.43	4.57	5.85	6.42	7.25	7.70
Gestation (days)	50	60	70	80	90	100	110	120	130	140

Table 1.4. Comparison of crown rump length with gestational age in fetal marmosets. Adapted from (Chambers and Hearn, 1985);(Hampton, 1971).

Gonadal formation and testicular development in the marmoset is postulated to take place during gestational weeks six to twelve (Li et al., 2005);(Hampton, 1971) (Table 1.5). During fetal gonad development the predominant cell type within the seminiferous tubule is reported to be the Sertoli cell with occasional germ cells (Hampton, 1971), as described in the human. At birth the testis has descended into an inguinal position (Dixson, 1993).

CRL (cm)	GA	Testis Shape	Cords/Tubules	Germ Cells
1.00	70	Oval	<20μm	No
2.00	80	Rounded	20-25μm	Rarely
3.50	90	Round	60-70μm	Few

Table 1.5 Features of gonadal development in the fetal marmoset. Adapted from (Hampton, 1971). GA – gestational age (approx.), CRL – crown rump length.

1.23.3. HPG axis in the marmoset

After birth the overall pattern of circulating gonadotrophins and testosterone is the same in the marmoset as in the human (Fig. 1.6), with an increase in levels of gonadotrophins and testosterone in the marmoset during the first 15 weeks of postnatal life (Dixson, 1986);(Mann and Fraser, 1996). This is followed by the childhood period which lasts from 15-45 weeks (Kelnar et al., 2002), during which levels of these hormones are low. The pubertal period between 45 and 75 weeks is associated with an increase in the levels of these hormones, which remain high in adulthood (Kelnar et al., 2002);(Chandolia et al., 2006);(Wistuba et al., 2006). Variability in the onset of puberty has been described between different colonies (Kelnar et al., 2002);(Chandolia et al., 2006).

A fundamental difference between the marmoset and the human surrounds the production of gonadotrophins. In the human, FSH and LH are produced from the pituitary gland. The marmoset produces FSH as in the human, but instead of LH the marmoset produces a chorionic gonadotrophin (CG) molecule. The marmoset does not possess the same LH receptor as the human due to its lack of an exon 10 (Zhang et al., 1997). This means that the receptor is unable to bind LH but instead will bind CG and cause an equivalent signal transduction to the human wild type LH receptor (Gromoll et al., 2000).

1.23.4. Germ cell development

In the marmoset, germ cell number varies with age. The neonatal period is associated with a 10-fold increase in germ cell number and a 5-fold increase in testis weight (Sharpe et al., 2003a). This study also described the predominant germ cell in the fetal testis as the gonocyte, based mainly on position in the cords and suggested that they undergo further differentiation and become prespermatogonia as in the human, based on the observation of a gradual shift from a central location within the seminiferous tubule to a position close to the basement membrane (Sharpe et al., 2003a). Once a point of contact is made with the basal lamina then the cells were

classified as prespermatogonia. In the neonatal marmoset at birth the majority (69%) of cells are gonocytes with the remainder being prespermatogonia. By 18-24 weeks the numbers of germ cells has risen dramatically but interestingly this was reported to include a gonocyte population (Sharpe et al., 2003a);(Chandolia et al., 2006). Another study had also described gonocytes within the seminiferous tubules of the marmoset at 3 months of age and reported this to be the predominant germ cell type at this age (reviewed in (Li et al., 2005)). The presence of gonocytes within the testis during the childhood period would not be expected in the human, however these marmoset studies were based on germ cell position and morphology described for human germ cells (Gondos, 1993), and did not include investigation of expression of proteins associated with germ cell differentiation that have already been described (section 1.14) and which can be used to characterize differentiation from gonocyte to spermatogonium in the human (Gaskell et al., 2004).

Based on morphological appearances spermatocytes are first observed between 21-43 weeks, coinciding with the hormonal rise at the onset of puberty (Chandolia et al., 2006);(Kelnar et al., 2002);(Wistuba et al., 2006). Studies in 35 week old marmsosets show that tubule lumens are beginning to form (McKinnell et al., 2001);(Kelnar et al., 2002) and occasional early spermatocytes are seen (Kelnar et al., 2002).

1.23.5. Postnatal Leydig cell development

Three types of Leydig cell have been described in the postnatal marmoset, which appear at different stages in life (Li et al., 2005). 3β-HSD is expressed at birth (Li et al., 2005), but increases during the neonatal period, with intense expression at 4 weeks of age (Kelnar et al., 2002). Expression of this steroidogenic enzyme is weak during the 'childhood' period (22 and 35 weeks), before increasing again in puberty and adulthood (Kelnar et al., 2002). This pattern of 3β-HSD expression mirrors the levels of testosterone during the neonatal, childhood, pubertal and adult periods as has been described (section 1.19.3).

1.23.6. Sertoli cell development

Sertoli cell numbers increase from the neonatal period to 18-22 weeks (Sharpe et al., 2003b). The timing of Sertoli cell proliferation in the neonatal period is similar to that seen in the human, whilst the predominant period of Sertoli cell proliferation in the rhesus monkey is peri-pubertal (Sharpe et al., 2003b). Androgen receptors can be detected by immunohistochemistry from 20 weeks onwards, and their detection coincides with the reduction in the intensity of AMH staining (Sharpe et al., 2003b).

1.23.7. Spermatogenesis in the marmoset

An adult pattern of spermatogenesis, with a full complement of germ cells from spermatogonia to fully formed elongate spermatids has been detected from 52 weeks of age in the marmoset (Chandolia et al., 2006). There are similarities in the structure and organisation of the seminiferous tubules exhibiting full spermatogenesis in the marmoset and human (Millar et al., 2000). Nine stages of spermatogenesis have been described in the marmoset (Holt and Moore, 1984). It has also been shown that the six stages of spermatogenesis in the human can also easily be applied to the marmoset (Weinbauer et al., 2001). Within each tubule cross-section in the marmoset, as in the human, there are between one and five stages observed (Fig. 1.15), whereas in rats and most other primates used for research, only a single stage of spermatogenesis will be seen in each cross section (Millar et al., 2000);(Weinbauer et al., 2001).

The spermatogonial sub-types are similar to the human with the presence of a single Type B spermatogonium compared to several types of spermatogonia in the rhesus monkey and rodent (Fig. 1.9). The efficiency of spermatogenesis can be estimated by the number of germ cells supported by a Sertoli cell and may also be influenced by the organisation of the seminiferous epithelium (Sharpe, 2003a). The germ cell to Sertoli cell ratio in the adult marmoset is 16:1 (Weinbauer et al., 2001) and the volume of germ cells supported per Sertoli cell in the marmoset is only 20% of

values found in the rat. This efficiency is similar to that described in the human (Sharpe et al., 2000).

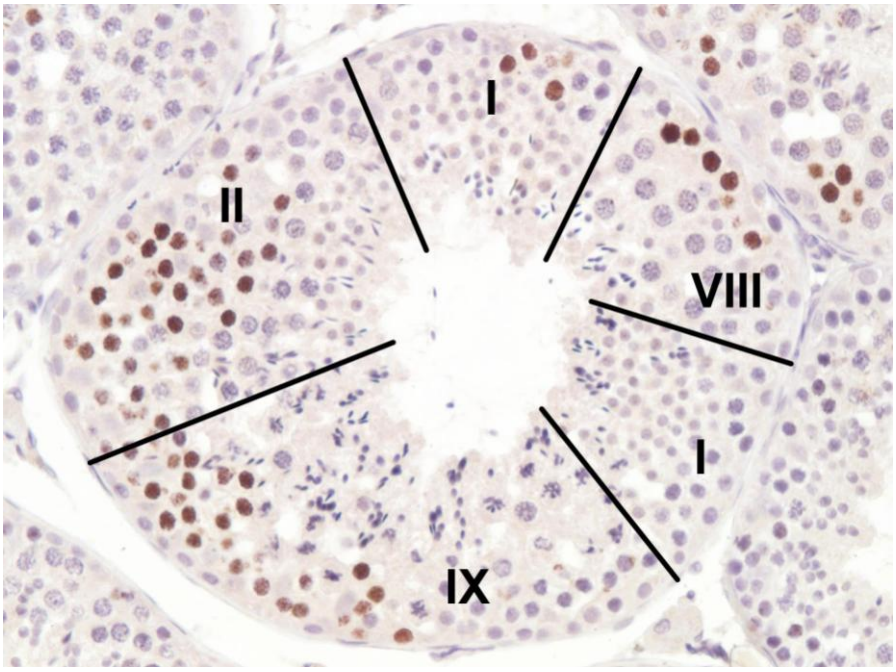

Figure 1.15. Cross section of a seminiferous tubule from an adult marmoset monkey. Several stages of spermatogenesis (indicated by numerals, corresponding to stage number) can be seen within the section. 5-bromo-2-deoxyuridine (BrdU; brown) staining also highlights variation in germ cell proliferation between the stages. Taken from (Millar et al., 2000).

1.23.8. Effects of GnRH suppression on testis development in the immature marmoset

1.23.8.1. GnRH antagonist treatment in the neonatal marmoset

GnRH antagonists (GnRHa) are peptides that bind with high affinity to the GnRH receptor in the anterior pituitary. They are non-functional and compete with the endogenous GnRH for the binding site and provide rapid sex steroid suppression (Griesinger et al., 2005). The neonatal testosterone rise can be abolished in marmosets by the administration of a GnRH antagonist for the first 14 weeks of postnatal life (Lunn et al., 1994). Treatment of neonatal marmosets with GnRHa results in a significant reduction in testis weight and a decrease in Sertoli cell

number at 18-22 weeks, which recovers by adulthood (Sharpe et al., 2000). This observation suggests that Sertoli cell proliferation can also occur in this species during the peri-pubertal period, which may also be the case for the human (Cortes et al., 1987);(Sharpe et al., 2003b). Germ cell numbers in neonatally treated marmosets are also reduced at 18-24 weeks, compared with controls, although this was variable and not statistically significant (Sharpe et al., 2003b). In adulthood there was no difference between germ cell volume per Sertoli cell in neonatally GnRHa treated animals compared to controls (Sharpe et al., 2003a). In addition to effects on germ cells and Sertoli cells, there is also a regression/suppression of maturation of the Leydig cells with GnRHa treatment, based on the ultrastructural appearance of the cells (Prince et al., 1998).

1.23.8.2. GnRH antagonist treatment in the juvenile marmoset

GnRH antagonists have also been used to study the role of the HPG axis in the juvenile marmoset testis (Kelnar et al., 2002). Both GnRHa treated and control animals had low or undetectable levels of serum testosterone between 25-35 weeks of postnatal age. GnRH antagonists resulted in a 34% decrease in testis weight, compared to controls. There was also delayed Sertoli cell function in terms of lumen formation and reduced Leydig cell volume, however the germ cell proliferation index, measured by PCNA expression in spermatogonia was not affected by GnRHa treatment (Kelnar et al., 2002). These results suggest that germ cell proliferation in the juvenile marmoset is independent of gonadotrophins and testosterone, although it must be remembered that this is the period when levels of gonadotrophins and testosterone are naturally low.

1.24. Testis xenografting

Removal of testis material and xenografting into a host animal provides an *in vivo* system to study the development of germ cells and/or intact testicular tissue. The technique has a number of potential applications in germ/stem cell biology, fertility preservation and the production of transgenic sperm (Dobrinski, 2008);(Wistuba

and Schlatt, 2002). Material for grafting may include cell suspensions or intact testis tissue and these may be grafted into a number of sites within the host animal including the testes themselves.

1.24.1. Spermatogonial transplantation

The use of spermatogonia for intratesticular transplantation was first reported by Brinster and colleagues in 1994. In the original study, predominantly spermatogonial germ cells were isolated from immature (4-12 dpc) donor mice and the cell suspension was then introduced into the seminiferous tubules of recipient mice depleted of endogenous germ cells using a micro-injection technique (Brinster and Zimmermann, 1994). Full spermatogenesis was restored in the transplanted testes (Brinster and Zimmermann, 1994). In addition the use of this technique produced spermatozoa that were capable of fertilisation and production of progeny (Brinster and Avarbock, 1994). Despite the success of spermatogonial stem cell transplantation between mice, there has been less success with xenografting of spermatogonia between other species. Full spermatogenesis has been achieved with transplants of spermatogonial stem cells from rats, hamsters and goats (Honaramooz et al., 2003);(Clouthier et al., 1996);(Ogawa et al., 1999) into a mouse host and the length of the cell cycle in these transplanted cells reflects the normal cell cycle of the donor species (Franca et al., 1998). However attempts to graft cells from more distant species, including primates often results in colonisation, but transplanted cells fail to develop beyond the stage of spermatogonia (Dobrinski et al., 1999) (Dobrinski et al., 2000) (Nagano et al., 2001). Transplantation of human spermatogonia into mice resulted in colonisation and proliferation in the first month, with no differentiation (Nagano et al., 2002), however longer term grafting resulted in no donor germ cells surviving (Reis et al., 2000). In addition the use of a GnRH agonist did not enhance spermatogenic recovery in these mice (Reis et al., 2000). It has been suggested that these spermatogonial stem cells fail to develop because murine Sertoli cells of the host are unable to provide appropriate factors

and a suitable niche to support development of the primate germ cells (Honaramooz et al., 2004).

There are limitations to this technique for use as an '*in vivo*' model of germ cell and testis development (Griswold et al., 2001). Firstly the system requires ablation of most of the endogenous germ cells in the testis of the host animal in order for the transplanted cells to colonise and develop. Mutant mouse strains such as the as W/W^{v} mutant that lacks Kit and hence does not have endogenous germ cells have been successfully tested as recipients and full restoration of spermatogenesis demonstrated (Lue et al., 2007). Alternatively endogenous germ cells can be removed from normal mice by treatment with the cytotoxic agent busulphan (Brinster et al., 2003) or radiotherapy (Honaramooz et al., 2005). However both these methods may result in alterations in the somatic compartment in the recipient (Zhang et al., 2007) reducing the success of the transplantation procedure since a compatible somatic compartment and the establishment of normal interactions between the spermatogonia and basement membrane appears to be necessary for germ cells to proliferate and differentiate (Rodriguez Sosa and Dobrinski, 2009);(Wistuba and Schlatt, 2002). Success of this technique also relies on the presence of spermatogonial stem cells in the germ cell suspension that is injected, however SSCs account for only one in every 3-4000 cells in the testis of an adult mouse (Tegelenbosch and de Rooij, 1993). The spermatogonial stem cell population can be enriched by using immature donors or by selection with fluorescent or magnetic cell sorting, based on the expression profiles characteristic of spermatogonial stem cells (Shinohara et al., 2000);(Hofmann, 2008);(Buageaw et al., 2005);(von Schonfeldt et al., 1999);(Hermann et al., 2009).

1.24.2. Grafting of testicular cell suspensions

In order to preserve compatability between the germ and somatic cells of the testis, cell suspensions can be grafted into the host animal (Arregui et al., 2008a). Cell suspensions containing mixed testicular cells from neonatal porcine and sheep testis have been used for subcutaneous xenografting and can result in the reformation of

cords and full differentiation of germ cells (Arregui et al., 2008a);(Honaramooz et al., 2007). The cell suspensions tend to develop more slowly than intact tissue grafts and this may be related to a reduced ability to synthesise testosterone (Arregui et al., 2008a). Dissociated neonatal rat testicular cells have also been used as single cell suspensions and cultured in 2D and 3D systems (Gassei et al., 2006). The 3D cultures in extracellular matrix formed regular spheres containing mixed cell types and a lumen. These structures contained putative germ cells and became more stable over time in culture. Subsequent xenografting into a nude mouse host resulted in further development of the tissue (Gassei et al., 2008). To date, the transplantation of isolated cell populations into nude mice has resulted in establishment of structures containing germ cells representative of full spermatogenesis in pigs, rodents and sheep. In rats, offspring have been produced from sperm taken from the transplanted material (Kita et al., 2007).

1.24.3. Testis tissue xenografting

The local environment and cell-cell relationships of the donor testis can be maintained by grafting whole tissue. This preserves the unique microenvironment for the cells within the tissue and in many species the tissue is responsive to host gonadotrophins and development of grafts was reported to be similar to that in normal testicular tissues (Rodriguez Sosa and Dobrinski, 2009). The importance of the niche is highlighted by the fact that transplantation of isolated germ cells from 12.5-dpc mice into host testes resulted in no differentiation, whilst grafting of whole testis tissue from the same age of fetus resulted in normal spermatogenesis (Ohta et al., 2004). Tissue can be grafted into various sites but is most frequently grafted subcutaneously under the dorsal skin of the host animal (Rodriguez Sosa and Dobrinski, 2009).

1.24.3.1. Choice of host mice for testis xenografting

Xenografting requires the use of immunocompromised host mice such as nude or SCID mice. No difference has been described for the success of grafting between

these two strains of host (Geens et al., 2006);(Watanabe et al., 2009). SCID mice lack T- and B-lymphocytes and some strains also lack complement and NK cells. Nude mice are homozygous for the nude gene, lack a thymus and therefore do not produce T-lymphocytes (Geens et al., 2006). This makes SCID mice more prone to infection and costly than the nude mice. In addition nude mice do not have hair, which may provide a more optimal temperature for subcutaneous grafting of testis tissue than the SCID mice.

1.24.3.2. Castration of host mice

Castration of the host animal is generally recommended (Rodriguez Sosa and Dobrinski, 2009). This reduces negative feedback on gonadotrophin secretion and results in LH stimulation of the grafts to produce testosterone. In grafts from bulls it has been reported that castration is not necessary as spermatogenic progression is not significantly different between castrate and intact hosts (Huang et al., 2008). This is in contrast to studies in rhesus monkey grafts, which report that castration is essential for development of the grafts (Honaramooz et al., 2004). Castration of the host animal means that the serum testosterone level and seminal vesicle weight of the host reflect graft testosterone production (Schlatt et al., 2002).

1.24.3.3. Success of xenografting of immature testis material in different species

Grafting of immature testis tissue into nude mice has resulted in the development of full spermatogenesis in the grafted tissue of several species including newborn mice (Honaramooz et al., 2002);(Schlatt et al., 2002);(Shinohara et al., 2002); hamsters (Schlatt et al., 2002); rabbits (Shinohara et al., 2002); pigs (Honaramooz et al.);(Zeng et al., 2009); goats (Honaramooz et al., 2002); bulls (Oatley et al., 2004); horses (Rathi et al., 2006); cats (Snedaker et al., 2004) and rhesus macaques (Honaramooz et al., 2004). In addition the spermatozoa isolated from grafts of mice, pigs, rabbits and rhesus macaques have all been shown to be capable of fertilisation using intracytoplasmic sperm injection (Honaramooz et al., 2008);(Schlatt et al.,

2003);(Shinohara et al., 2002). In porcine grafts, 24% of spermatozoa were fertilisation competent and 8% resulted in the formation of blastocysts (Honaramooz et al., 2008), whilst in rhesus monkeys 80% of the spermatozoa appeared viable and ICSI resulted in 3 out of 16 injected oocytes developing to the blastocyst stage (Honaramooz et al., 2004). Mouse xenografts have resulted in the production of spermatozoa that are capable of producing progeny (Schlatt et al., 2003). Fertilising ability of these sperm was similar to spermatozoa extracted from normal mice and 7 pups were produced from 312 manipulated oocytes (Schlatt et al., 2003). The phenomenon of accelerated spermatogenesis has been demonstrated in grafts from bovine and monkey (Honaramooz et al., 2004);(Schmidt et al., 2006b), whilst grafts from cats may exhibit normal (Snedaker et al., 2004) or even decelerated development (Kim et al., 2007). This is particularly important as some of the donor species (i.e. rhesus macaques) do not naturally reach full spermatogenesis until 4 years of age, which is greater than the lifespan of a potential recipient mouse (Honaramooz et al., 2004). Transplantation of immature testis tissue from 13 month old rhesus macaques, subcutaneously onto the back of ICR/SCID mice resulted in accelerated spermatogenesis with production of mature sperm within 7 months. This was in comparison with testicular development in 2 year old controls where no germ cell differentiation was observed (Honaramooz et al., 2004).

Post-meiotic differentiation of germ cells in grafts from the horse (Rathi et al., 2006) and rhesus monkey are enhanced and accelerated by the administration of exogenous gonadotrophins to immature host mice (Rathi et al., 2008);(Honaramooz et al., 2004) and the somatic cell development still occurs despite the shortening of the childhood 'quiescent' period of testis development (Rathi et al., 2008).

Despite the success of using grafting of testicular tissue from some species there are species in which the efficiency of spermatogenesis remains low. They include bovine (Rathi et al., 2005), equine (Rathi et al., 2006), cat (Snedaker et al., 2004) and monkey (Honaramooz et al., 2004) grafts. The marmoset is another species in which

full spermatogenesis is not induced after grafting (Schlatt et al., 2002) with a failure of germ cells to develop beyond the spermatocyte stage. This was associated with low androgen levels produced by the graft in the castrated host. In an attempt to overcome this problem, further experiments were performed using co-grafting of hamster testicular tissue, which is known to produce androgen in the host. The deletion of exon 10 in the LH receptor gene was also addressed by grafting with the addition of hCG treatment (Wistuba et al., 2004), which is known to act on the marmoset LH receptor to produce testosterone (Kholkute et al., 1983). Despite these modifications there was no effect on the outcome of the marmoset grafts. Indeed when testis tissue was grafted autologously into newborn marmosets the same meiotic arrest occurred along with a reduction in testosterone (Wistuba et al., 2006). The cause of the failure to induce full spermatogenesis in marmoset grafts was hypothesized to be related to hyperthermia, decreased testosterone levels or the multistage organisation of the seminiferous epithelium. Further studies have been performed using autologous grafts in marmosets placed either ectopically (under the back skin) or orthotopically (into the scrotum) (Wistuba et al., 2006);(Marc Luetjens et al., 2008). This resulted in complete spermatogenesis in the orthotopic grafts compared to the meiotic arrest in ectopic grafts as seen previously. Hyperthermia has therefore been suggested as the reason for lack of success of ectopic grafting but further studies are required to validate this hypothesis.

1.24.3.4. Xenografting of immature human testis tissue

Grafting of human testicular tissue was first reported over thirty years ago (Povlsen et al., 1974);(Skakkebaek et al., 1974) with the grafting of fetal human tissue subcutaneously into the lateral abdominal wall of nude mice. After 4-8 weeks grafts had become richly vascularised and contained seminiferous tubules with recognisable gonocytes and undifferentiated Sertoli cells. Since these original studies were reported only one further study has been published (Yu et al., 2006); testes from two human fetuses (20 and 26 weeks gestation) were grafted for 116-135 days. The grafts were examined histologically and the cords had formed lumina

with accelerated maturation of Sertoli cells and the presence of spermatogonia was noted (Yu et al., 2006). No data relating to proliferation or differentiation of germ cells within human fetal testis xenografts has been reported to date

Human testis xenografts have also been performed using pre-pubertal testis tissue (Goossens et al., 2008);(Wyns et al., 2008);(Wyns et al., 2007). In the study from Gossens *et al*, testis biopsy material from pre-pubertal patients with sickle cell anaemia was grafted into nude mice and allowed to develop for 4 or 9 months. The authors reported survival of tubules and were able to detect Sertoli cells by staining with vimentin as well as occasional tubules containing germ cells that expressed the spermatogonial protein MAGE-A4 (Goossens et al., 2008). Survival and proliferation of spermatogonia has also been demonstrated in short term grafts taken from cryptorchid testes in boys aged 2-12 years. Longer term germ cell survival was demonstrated in testis tissue from 5 boys aged 7-14 years, grafted for 6 months by investigating MAGE-A4 expression in the grafts (Wyns et al., 2008). Cells within these grafts were also proliferating, as demonstrated by Ki67 expression. In addition, this study reported the presence of primary spermatocytes, some of which were entering meiosis. There were no spermatids, although in one graft from a 14 year old boy there were cells resembling spermatozoa with abnormal appearance (Wyns et al., 2008).

1.24.3.5. Effect of donor age on xenografting success

Donor age has been shown to affect the success of cell survival in grafted material in several species (Schmidt et al., 2006b) and the presence of mature germ cell types has been shown to result in generally poorer graft development (Rathi et al., 2006);(Huang et al., 2008). Grafting of prepubertal mouse testis tissue is successful and full spermatogenesis has been reported, but when adult testis tissue was grafted success rates fell (Geens et al., 2006). Attempts to graft testes from adult pigs, goats, cattle and rhesus monkey resulted in degeneration of tubules in the surviving grafts (Arregui et al., 2008b). This has also been demonstrated in autologous grafts from

adult marmosets in which grafts did not survive (Marc Luetjens et al., 2008). Attempts to graft human adult testis has also been unsuccessful (Schlatt et al., 2006) and although occasional surviving spermatogonia have been described in 21-23% of the 74 grafts, no meiotic progression occurred. Most of the grafts had either sclerosed or were Sertoli cell only (SCO) (Geens et al., 2006). Spermatogonial stem cell survival was occasionally seen in human adult grafts when spermatogenesis had been suppressed prior to grafting with GnRH (Schlatt et al., 2006). The maximum practical age for successful xenografting appears to be just before puberty (Kim et al., 2007) and the success of differentiation in the xenografts from many species appears to depend upon the intensity/efficiency of spermatogenesis at the time of grafting (Arregui et al., 2008b), with improved survival and differentiation when the intensity/efficiency of spermatogenesis is lower (Arregui et al., 2008b).

The failure of adult grafts to re-establish spermatogenesis has been attributed to several possible factors including cessation of Sertoli cell proliferation, ischaemia and failure of angiogenesis (Rodriguez Sosa and Dobrinski, 2009). Angiogenesis has been shown to be important because blood vessel numbers are significantly increased in grafts containing functional tubules compared to those with non-functional tubules (Schmidt et al., 2006a). Vascular endothelial growth factor (VEGF) has been shown to increase the size of bovine xenografts in addition to increasing the numbers of elongate spermatids (Schmidt et al., 2006a). However the number of blood vessels was not increased by this treatment, suggesting that VEGF was not exerting this effect by increasing angiogenesis.

1.25. Aims of the thesis

The reproductive potential of the male depends on the normal development of the testis. This process continues throughout life from the fetal period into adulthood. Normal development of the germ cells is crucial to this process as these cells will ultimately become the male gametes. The development of the germ cells is dependent on their relationship with the supporting Sertoli cells and Leydig cells

that provide hormonal factors required for normal development. The term TDS has been used to describe a group of conditions that appear to have a common origin in fetal life and affect the cellular interactions that occur during testis development. These conditions include TGCT and infertility.

This thesis aimed to investigate the origins of TGCT by characterizing the phenotypes of the pre-neoplastic CIS cells and comparing them to the associated supporting Sertoli cells and also to the germ cells of the normal human fetal testis. These phenotypes were also investigated in relation to the proliferation status of germ cells/CIS in order to identify whether certain phenotypes may influence the invasive potential of the CIS cells (chapter 3). The second aim was to investigate the Common Marmoset monkey as a potential animal model of germ cell development that could be relevant to conditions such as CIS and TGCT. This concept was based on its known similarity to the human in many other aspects of reproductive biology and testis development (chapter 4). Having established the animal model the next aim was to develop an in vivo xenografting system that could be applied to the human and marmoset to recapitulate normal testis development (chapter 5). Finally, manipulation of normal development of the testis in these model systems was attempted using substances known to suppress normal hormonal activity (GnRH antagonists) or to induce features of the TDS in a rodent model (phthalates) to provide insight into clinical disorders of testis development, in particular TGCT.

2 General materials and methods

2.1. Human tissue

2.1.1. Human fetal testis tissue

Human fetal testis tissue was obtained from elective terminations of pregnancy during the first and second trimesters. Women gave consent in accordance with national guidelines (Polkinghorne) and the terminations were carried out by medical and nursing staff at the Simpson's Centre for Reproductive Health, within the Royal Infirmary of Edinburgh. Termination of pregnancy was induced with mifepristone (200 mg, orally), followed by misopristone (Pharmacia, Surrey,UK. 200 mg every 3 hours per vaginam). No terminations were due to fetal abnormalities. Gestational age was determined initially by ultrasound examination, followed by direct measurement of foot length for 2nd trimester samples. Ethical approval was obtained for the use of fetal human tissue from the Lothian Research Ethics Committee (REC reference number: 08/S1101/1).

2.1.2. Human testicular germ cell tumours

Human testicular germ cell tumour samples were obtained from the archive in the pathology departments of the Western General Hospital and the Royal Infirmary of Edinburgh with ethical approval from the Lothian Research Ethics Committee (REC reference number: 07/S1101/8) and NHS R&D approval (R&D number: 2006/R/RM/20). Slides stained for diagnostic purposes with haematoxylin and eosin were examined by myself, under light microscopy for evidence of the pre-neoplastic lesion carcinoma in-situ. Archived paraffin embedded blocks corresponding to these slides were located and sectioned (2.8.2) for immunohistochemical analysis.

Sections of testis material from pre-pubertal patients containing pre-invasive carcinoma in-situ were kindly supplied by the Department of Pathology, Erasmus MC – University Medical Center, Rotterdam, Netherlands courtesy of Professor Leendert Looijenga.

2.2. Animal work

All animal work was performed in accordance with the UK Home Office Animal Scientific Procedures (UK) Act (1986) under project licences 60/3544 and 60/219. Day to day husbandry duties for work with nude mice was carried out by University of Edinburgh animal technicians. Surgical procedures and treatments of nude mice were carried out by myself under Home Office personal licence 60/11139. Husbandry duties for the Wistar rats were carried out by Mark Fisken in the HRSU animal facility. Work with common marmoset monkeys was performed by the staff at the Medical Research Council primate facility, in particular Keith Morris and Jim Macdonald. This included general husbandry, treatments and tissue retrieval.

2.3. Animal welfare

All animals used were maintained in our own animal facilities according to UK Home Office guidelines. Common marmoset monkeys (*Callithrix jacchus*) used in this study came from a closed colony that has been self-sustaining since 1973. Wistar rats were bred within our own animal facility and were housed in solid bottomed cages, typically in groups of six with stud males housed individually. Water and food was available *ad libitum*. Light was provided from 07:00 – 19:00 and the temperature was maintained at 20-25^{o}C, with humidity of 55%. Male CD1 Nude (Crl:CD-1 -Foxn1^{nu}) mice were obtained from Charles River UK (Margate, England). These mice are immunodeficient, lacking a thymus and T-cells, making them a suitable animal host for xenografting as they do not reject the grafts. Mice were housed in individually ventilated cages (IVCs; Tecniplast, Varese, Italy). Bottled sterile water and sterile food was available *ad libitum*. Light was provided from 07:00 – 19:00 and the temperature was maintained at 20-25^{o}C, with humidity of 41%. The animals were housed in groups of three initially, but were individually housed following xenografting surgery. Neonatal mice from a mixed background were used for initial allografting studies. These mice were kindly provided by Dr Lee Smith, HRSU.

2.4. Tissue collection

2.4.1. Marmoset testes

Testes were obtained from the fetuses of elderly pregnant marmosets in the colony breeding stock that were being euthanised to make way for younger breeding animals. Gestation in the marmoset lasts for approximately 20 weeks (144 days) (Windle et al., 1999). Regular, systematic palpation and/or ultrasound were used to diagnose pregnancy and estimate gestational age and this was performed by Keith Morris in the MRC primate facility. Experience has shown this to be accurate to within one week and confirmed by reference to published crown rump length (CRL) data (Chambers and Hearn, 1985) as seen in Table 1.4. Mothers and fetuses, or postnatal animals were euthanised by injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Merieux Ltd, Harlow, Essex, UK). The fetuses were delivered by hysterotomy. Fetuses were fixed in Bouins for 6 hours (larger fetuses were partially dissected prior to fixing) and then transferred to 70% ethanol. Sections of postnatal testes were obtained from tissue remaining from previous studies (Kelnar et al., 2002) (Sharpe et al., 2003a) in order to reduce the animal numbers required. In addition, testes were also obtained from newborn animals euthanized for colony management purposes (triplet births). All testes were fixed as described above, prior to processing. For xenografting experiments a small piece of testis tissue was fixed in Bouins as described above and the remainder was placed into media and kept on ice until grafting.

2.4.2. Human testes

2.4.2.1. First and second trimester human fetal testes

Human fetal testes were obtained from 1st (8-10 weeks) or 2nd (14-19 weeks) trimester human fetal testes. The sex of first trimester samples was determined by PCR for the SRY gene as previously described (Gaskell et al., 2004). Testes were fixed for 2h in Bouins and then transferred into 70% ethanol prior to processing. For xenografting experiments a

small piece of testis tissue was fixed in Bouins as described above and the remainder was placed into media and kept on ice until grafting.

2.4.2.2. Third trimester and postnatal human testes

Third trimester and post-natal human testes were obtained at autopsy with consent of the legal guardian (courtesy of Dr Katherin McKenzie, Consultant Pathologist, Pathology Department, Royal Infirmary of Edinburgh). Testes were from boys who died from various causes (excluding reproductive and endocrine abnormalities). Testes were fixed in 10% Neutral Buffered Formalin for at least 24h and processed as below.

2.4.3. Rat testis tissue collection

Pregnant dams were killed by inhalation of carbon dioxide when their fetuses had reached embryonic day 15.5 (e15.5, n=5) or embryonic day 19.5 (e19.5, n=5). Fetuses were removed, decapitated and placed in ice-cold phosphate buffered saline (PBS; Sigma, Poole, Dorset, UK). Testes were removed via microdissection and fixed for 1h in Bouins followed by transfer into 70% ethanol. I am grateful to Matthew Jobling for providing me with the dissected rat testis tissue.

2.4.4. Mouse testis tissue collection

Newborn mice were killed by decapitation and the testes were removed. A small piece was fixed for 2h in Bouins and then transferred into 70% ethanol prior to processing. Testes for use in xenografting studies were placed directly into media and kept on ice until grafting.

2.5. Animal treatments

2.5.1. GnRH antagonist treatment

Newborn marmosets were treated by subcutaneous injection with 10mg/kg of a potent long-acting GnRH antagonist (GnRHa; Antarelix; Europeptides, Argentueil, France) weekly for four weeks to suppress the neonatal testosterone surge (Sharpe

et al., 2003b);(Lunn et al., 1994). Each animal had a co-twin acting as a direct control. These co-twins were treated with vehicle instead of GnRHa.

2.5.2. Monobutyl phthalate (MBP) treatment

Nine pregnant females were administered 500 mg/kg/day MBP (TCI Europe, Zwijndrecht, Belgium) by oral gavage from 7–15 weeks of gestation. MBP was made up by mixing with corn oil. A total of 11 male offspring originated from these treated mothers and 6 of these were killed at birth (1–5 days), whilst 5 were allowed to grow into adulthood.

2.6. Animal Surgical Procedures

2.6.1. Preparation of surgical area and anaesthetic equipment

Nude mice (Charles River Laboratories, Margate, UK) were used as hosts for all xenografting experiments. A sterile surgical field was prepared inside a laminar flow cabinet. The cabinet and all equipment were cleaned with antiseptic wipes (Trigene, Medichem International Ltd, Sevenoaks, UK) and a warming mat covered in a plastic disposable sterile drape was placed inside the cabinet. An anaesthetic rig (Vet Tech Solutions Ltd, Congleton, UK) was set up to direct anaesthetic to a chamber for induction of anaesthesia and the anaesthetic chamber was filled with Isoflurane. The anaesthetic was then switched to tubing ending in a nosepiece, which was attached to the sterile field with a clamp; for maintenance of anaesthesia during surgery.

2.6.2. Castration of nude mice

Isoflurane (5%) and oxygen (2L/min) were directed into an anaesthetic box, which had been placed into the laminar flow cabinet. Mice were put into the box until anaesthetised. They were then transferred onto the sterile field and placed supine with their noses directed into the nosepiece of the tubing for maintenance of anaesthesia and the gases were directed into this tubing with the concentration of

Isoflurane reduced to 2%. The perineum was sprayed with 70% ethanol to clean the surgical area. A longitudinal scrotal incision was made using surgical scissors followed by an incision in the underlying tunica. The testis, spermatic cord and testicular fat pad were exteriorised and the spermatic cord was tied off using Mersilk 3.0 silk sutures (Ethicon, Livingston, UK) and severed distal to the suture. The skin was sutured closed using 3.0 sutures. The process was then repeated for the second testis (Fig. 2.1).

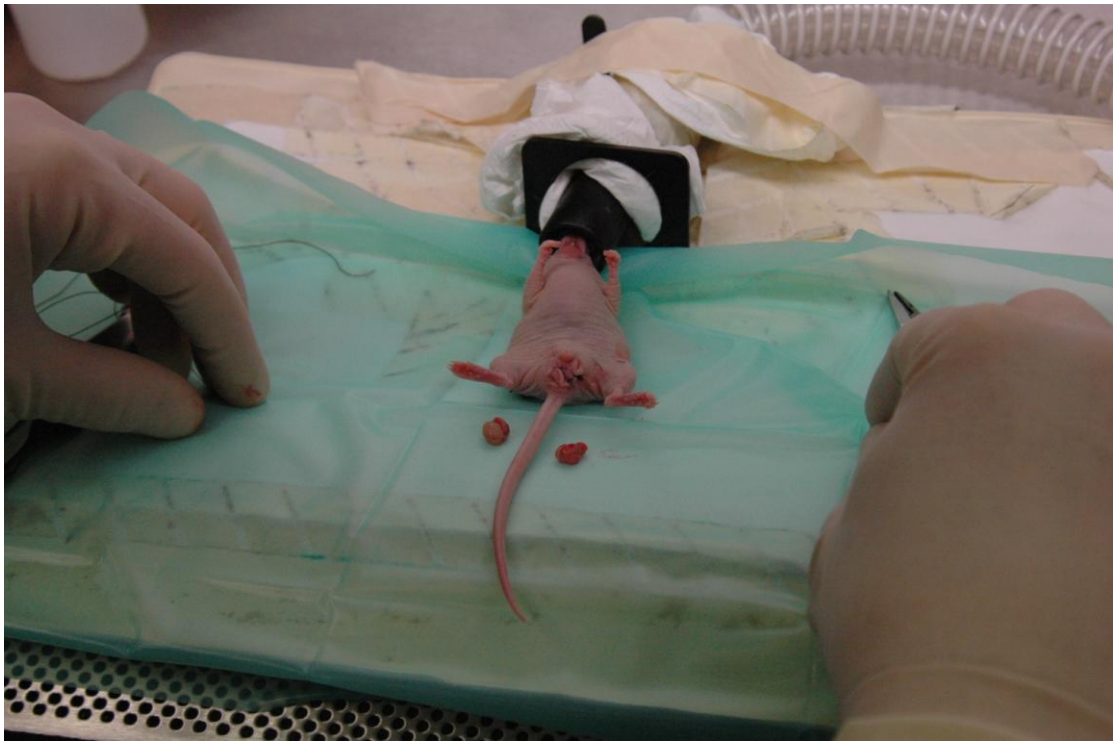

Figure 2.1. Castration of a nude mouse host prior to xenografting. Note the set up of the anaesthetic delivery and sterile field.

2.6.3. Preparation of donor testis material for xenografting

Donor testes were placed immediately into ice cold media containing Liebowitz L-15 with glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids (all obtained from Sigma, Poole, UK). Prior to grafting the testes were placed into a petri dish and cut into small (1mm^3 approx.) pieces using sterile scissors (Fig. 2.2).

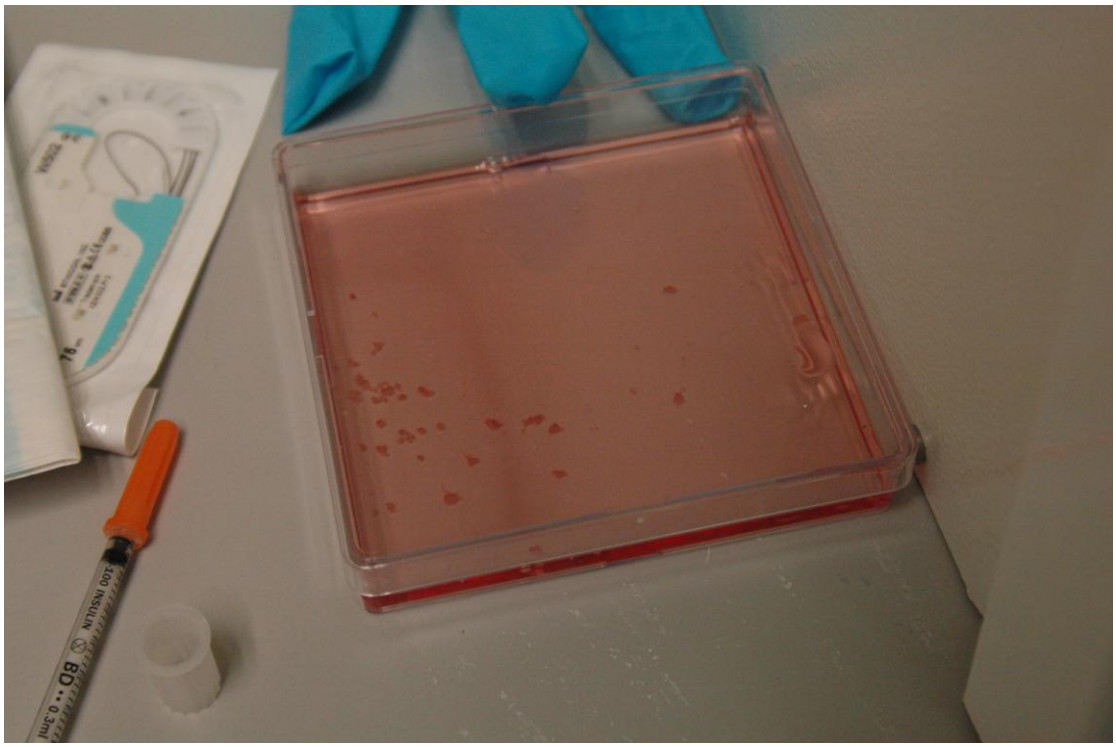

Figure 2.2. Preparation of the donor testis for xenografting. The testis has been cut into small pieces with sterile scissors (1mm^3 approx.).

2.6.4. Genotyping for *SRY* in first trimester human fetal gonads

It was not possible to determine the sex of the first trimester fetuses for xenografting by microscopy and therefore tissue was taken from the fetus and genotyping for the *SRY* gene was performed. A small piece of fetal tissue (e.g. hand/foot) was digested in 100µl 25mM NaCl/0.2mM EDTA (Digest buffer 1) at 95°C for 20min, followed by neutralisation in 100µl 40mM Tris.HCl, pH 7.5 (Digest buffer 2). The samples were vortexed for 30s and the reaction mixture (total volume of 25µl) was made up as follows:

5µl DNA (from samples)
1µl PCR buffer (Qiagen, Crawley, UK)
1µl dNTP's (Qiagen)
0.5µl SRY forward primer

0.5µl SRY reverse primer

15.375µl dH_2O (Qiagen)

0.125µl Taq polymerase (Qiagen)

The primer sequences were as follows:

SRY-F: ACAGTAAAGGCAACGTCCAG

SRY-R: ATCTGCGGGAAGCAAACTGC

Samples were placed in a Perkin Elmer PTC-100 (Perkin Elmer, Massachusetts, USA) programmable thermal cycler PCR machine and incubated according to the following conditions:

95°C for 15 min	
95°C for 30s	35 cycles
58°C for 30s	
72°C for 45s	
72°C for 10 min	

Loading dye (10X, Promega, Southampton, UK) was added to the samples. A 2% agarose gel in TE buffer was prepared containing 1:10000 GelRed DNA stain (Biotium, California, US). The gel was placed into an electrophoresis tank (Bio-Rad, Hemel Hempstead, UK) containing TE buffer and 10µl samples were loaded into the wells. A 100bp ladder (5µl; Biolabs, Hitchin, UK) was placed in the wells either side of the samples. Samples were run at 85V for 1 hour and visualised under UV light using a Geneflash Syngene bioimaging UV light box (Syngene, Cambridge, UK) and photographed. A 300bp product indicated the presence of the SRY gene and identified the samples as male. Some of the genotyping was carried out by Dr Andrew Childs, HRSU, who was using tissue from these samples, and I am grateful for his assistance.

2.6.5. Xenografting of testis material into nude mouse hosts

Anaesthetised mice were placed prone and their dorsal skin sprayed with 70% ethanol. Pieces of donor testis material were placed into the tip of a 13G cancer implant needle (Popper and Sons, New York, USA) with the trochar withdrawn. The needle was inserted 1-2cm under the back skin and the trochar pushed through the needle to implant the tissue. The needle was withdrawn and the puncture site was held closed for 1-2 seconds. Up to six grafts (three either side of the midline) were inserted into each recipient (Fig. 2.3). A subcutaneous injection of 4mg/kg (0.015mls of 5mg/ml) carprofen diluted in sterile water for injection (Rimadyl SA; Pfizer, New York, USA), was given into the lower right flank of each mouse for analgesia. The mice were returned to IVCs and housed individually. Rimadyl LA (0.5mls per 250mls sterile water) was added to the drinking water for 5 days post surgery and, if antibiotics were required, Baytril (Enrofloxacin; Bayer, Germany 1ml per 250mls sterile water) was also added to the drinking water. I am grateful to Dr Stefan Schlatt, Institute of Reproductive and Regenerative Biology, Munster, Germany, for providing training in how to undertake the testis xenografting technique.

Figure 2.3. Subcutaneous xenografting procedure. Testis tissue pieces were placed into the bevel of the needle and injected under the skin (upper panel). Positions of graft placement can be seen in the lower panel.

2.6.6. Treatments for xenografted mice

2.6.6.1. hCG treatment

hCG treatment was used to stimulate the production of testosterone from the testis grafts. A vial of 5000IU hCG (Pregnyl, Organon Laboratories, Cambridge, UK) was diluted to 1 ml with 0.9% saline. 1% Fetal calf serum (Sigma) was added to allow storage of hCG aliquots. Aliquots of 25 IU (5µl) were stored at -20°C. Vehicle treatments consisting of 5µl aliquots of 0.9% saline with 1% fetal calf serum were also stored at -20°C. Animals were randomly assigned to receive hCG or vehicle. Immediately prior to use, aliquots were diluted in 0.25mls sterile 0.9% saline in 1ml plastic syringes and 0.20mls (20IU) was injected subcutaneously into the scruff of the neck using a 25G injection needle. Injections were given within a laminar flow

unit. Treatment began one week after grafting and continued three times a week for the duration of the grafting period.

2.6.6.2. Di (n-butyl) phthalate (DBP) treatment

Some of the grafted mice received DBP treatment. In addition, some of these mice also received hCG as described in the previous section. DBP was made up by mixing DBP (Sigma) with corn oil. To make up the DBP, 0.5mls DBP was added to 9.5mls corn oil to give a concentration of 50mg/ml. Two regimens were used. In the first regimen, host mice received 350µl (500mg/kg) DBP by oral gavage 6 hours prior to retrieval of the grafts. In the second regimen the mice received the same dose on the four consecutive days prior to graft retrieval. For both regimens, vehicle treated controls were used. These control hosts received the same volume of corn oil without DBP.

2.6.7. Retrieval of testicular xenografts

Grafts were retrieved after the specified grafting period. The mice were killed by cervical dislocation under Schedule 1 of the Animal (Scientific Procedures) Act 1986. Body weight was recorded using an electronic analytical balance (Handy H110, Sartorius) and blood was obtained by cardiac puncture for measurement of serum testosterone levels. The seminal vesicles were dissected out and weighed as a surrogate marker of graft testosterone production. The back skin was then peeled back to reveal the subcutaneous tissue. The grafts were easily identified as discrete masses with a loose adherence to the underside of the skin (Fig. 2.4). These grafts were dissected and weighed before being placed into Bouins fluid for further analysis.

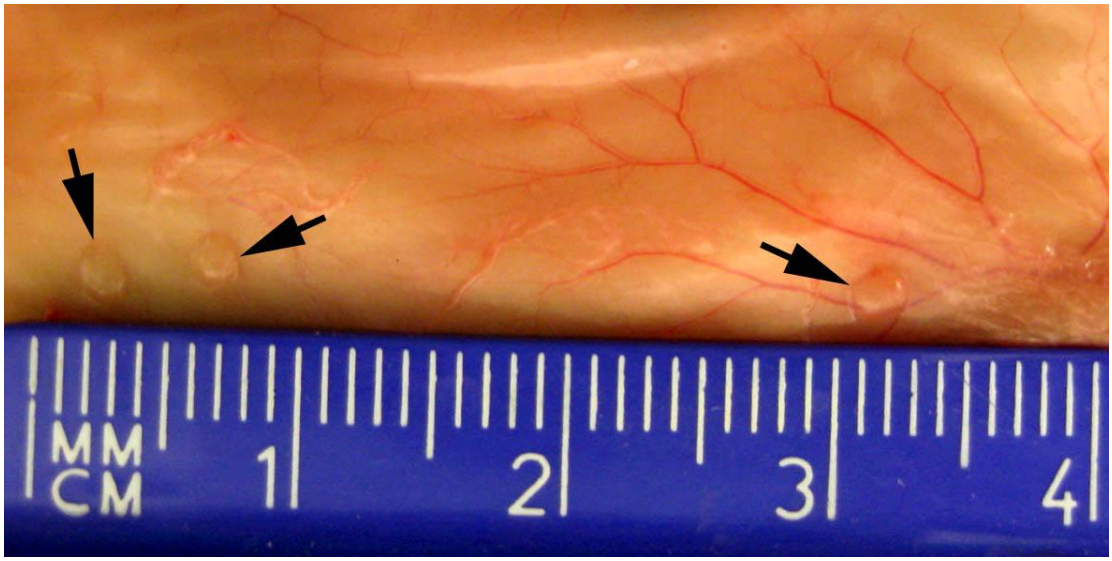

Figure 2.4. Retrieval of testicular xenografts. Testicular xenografts (arrows) on the undersurface of the back skin of a nude mouse host.

2.7. Serum testosterone radioimmunoassay

A competitive radioimmunoassay (RIA) was performed using a known concentration of radiolabelled antigen (testosterone labelled with I^{125}, MP Biomedicals, UK) against unlabelled endogenous testosterone present in the sample. The radiolabelled testosterone and the testosterone within the sample compete for antibody binding sites. The free (unbound) radiolabelled testosterone is inversely proportional to the amount of testosterone in the sample. Measuring free radiolabelled testosterone can then be used to calculate the level of testosterone in the sample. Radiolabelled testosterone was added to each sample prior to the addition of primary antibody (AMS Biotech, Abingdon, UK), used at 1:600,000. The primary antibody was incubated for 3 hours at room temperature. The samples were then incubated overnight at 4°C with a secondary donkey-anti-rabbit antibody. The secondary antibody binds to the testosterone/antibody complex to form a stable precipitate. Then 1ml of wash buffer was added and the tubes centrifuged for 30 minutes at 3000rpm. The supernatant was then decanted and the amount of radiation in the precipitate was measured and compared to a standard curve of known testosterone concentrations. A gamma counter (WIZARD 1470,

Perkin Elmer, Turku, Finland) was used to measure residual I^{125}. This assay has a sensitivity of 0.1ng/ml. The Intra assay coefficients of variation (%) are 6.0, 5.5 and 6.6, whilst Inter assay variation (%) is 14.2, 8.7 and 7.9. In each run, method blanks, quality control samples (MP Biomedicals, Solon, Ohio, USA), and standards were analysed alongside the unknown samples. Results were analysed with AssayZap (Biosoft, Cambridge, UK) and data expressed as ng testosterone per 1ml. I would like to thank Nancy Evans, from the Assay lab, HRSU, Edinburgh for performing the testosterone assays.

2.8. Tissue preparation for staining procedures

2.8.1. Fixed tissue processing

After fixing in Bouins, testis tissue was transferred to 70% ethanol and subsequently processed by staff in the MRC HRSU histology department. The tissue was processed through a series of graded alcohols using a Leica TP-1050 processor (Leica UK Limited, UK), before being embedded into molten paraffin wax and loaded onto plastic cassettes. Once processed, blocks were stored at room temperature.

2.8.2. Tissue sectioning

Blocks of paraffin embedded tissue were chilled on ice prior to sectioning. Sections (5μm thickness) were cut using a hand operated microtome (RM 2135, Leica) and floated onto warm water (45-50^{o}C) in a water bath. Individual sections were then mounted onto charged glass slides (SuperFrost, Menzel GmbH & Co.) and dried overnight at 50^{o}C.

2.8.3. Dewaxing and re-hydration

Slides were de-waxed sequentially in xylene for 5 minutes (x2), followed by graded alcohols: absolute (x2), 95%, 80% and 70% for 20 seconds each. The slides were then washed in tap water.

2.9. Haematoxylin and eosin staining

Following dewaxing and rehydrating, sections were submerged in haematoxylin (Triangle Biomedical Sciences, Durham, USA) for 6 minutes. Sections were rinsed in tap water and briefly placed into 1% acid alcohol for between 1 and 5 seconds until the desired staining was achieved. Sections were then washed in tap water and placed in Scott's tap water for 30 seconds to stain the nuclei blue, followed by a rinse in tap water. The sections were then immersed in eosin Y (1% aqueous solution mixed with 1% alcohol solution at a ratio of 3:1; Triangle Biomedical Sciences) for 20 seconds and washed again in tap water. The sections were then dehydrated in a series of alcohols 70%, 85%, 95%, absolute, absolute for 20 seconds each and then placed in xylene for 5 minutes. The sections were then mounted under glass coverslips (VWR International, Lutterworth, UK) with Pertex mounting medium (Cell Path, Hemel Hampstead, UK).

2.10. Immunohistochemistry

2.10.1. Immunohistochemistry method

Immunohistochemistry was performed by the indirect immunoenzyme method (Fig. 2.5). This technique involves the application of an unlabelled primary antibody that reacts specifically to the antigen within the tissue. A biotin labelled secondary antibody directed against IgG of the species that the primary antibody was raised against is then applied. A streptavidin conjugated enzyme layer is then added, which binds to the biotin from the secondary layer and finally the substrate for the enzyme is added. The streptavidin is derived from streptococcus avidini and is uncharged, which means that unlike avidin there is no non-specific electrostatic binding to the tissue. This means that the method is more sensitive than the Avidin-Biotin Complex (ABC) method.

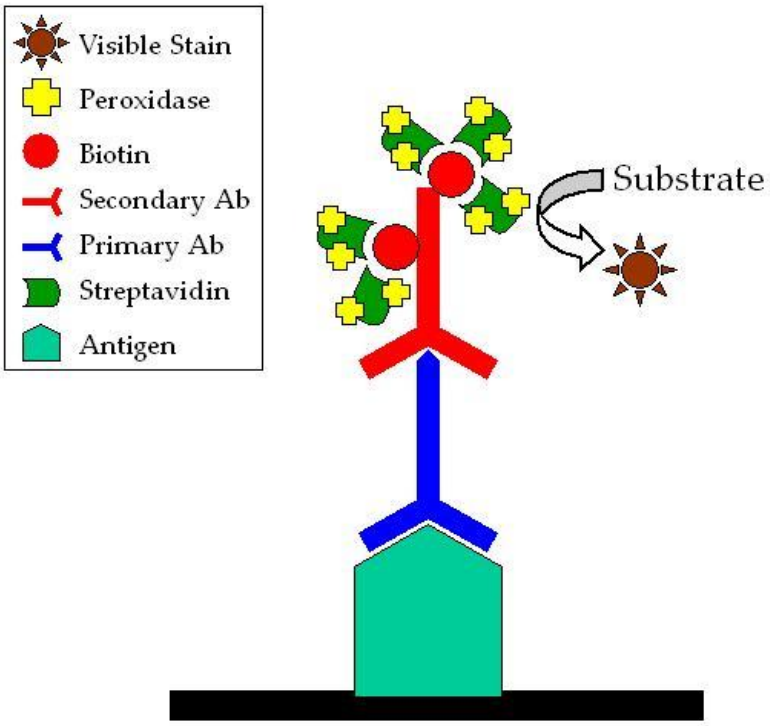

Figure 2.5. Indirect immunohistochemistry. Sequential primary and secondary antibodies are applied. Peroxidase labelled Streptavidin binds to biotin on the secondary antibody and the peroxidise enzyme catalyses the substrate reaction to produce a visible colour.

2.10.2. Single staining

Sections were prepared as described (2.8.1 – 2.8.3)

2.10.2.1. Antigen retrieval

Antigen retrieval was required to unmask some of the antigens (Table 2.1) because the fixation process may result in protein cross-links within the tissue, which would otherwise prevent binding of the antibody to the antigen (Norton et al., 1994). Dewaxed and rehydrated slides were immersed in 2L of boiling citrate buffer (made up 1:10 with distilled water) within a Tefal Clipso pressure cooker (Tefal, Slough, UK) and set to the highest pressure setting. The slides were left for 5 minutes on full pressure, as indicated by the continuous release of steam from the valve. The pressure was then released and the pressure cooker taken of the heat and slides left

in the hot citrate for a further 20 minutes. Slides were then removed and cooled in water.

2.10.2.2. Blocking

Endogenous peroxidise activity was blocked by immersing slides in 3% (v/v) hydrogen peroxide (VWR), followed by rinsing in water. The slides were then washed in TBS for two 5 minute periods. Slides were wiped dry and a circle drawn around the tissue with an ImmEdge hydrophobic pen (Vector Laboratories, Inc. Burlingame, CA) to allow good coverage of the tissue with the subsequent reagents. For human and primate tissue, which may contain endogenous biotin, streptavidin and biotin blocks were applied using a blocking kit (Vector Laboratories Inc.). Each block was applied (1 drop per slide) for 15 minutes with brief TBS washes in between. The final blocking step was with normal serum for 30 minutes, diluted 1:5 with TBS and containing 5% (w/v) bovine serum albumin (BSA; Sigma). Choice of serum (normal serum/TBS/BSA) depended on the species in which the secondary antibody was raised.

2.10.2.3. Primary antibodies

Primary antibody diluted in normal serum/TBS/BSA was then applied to the sections and incubated overnight at 4^{o}C. Details of primary antibodies used for single immunohistochemistry are detailed in Table 2.1.

Antigen	Source	Species	Dilution	Retrieval
3β-HSD	Gift[a]	Rabbit	1:1000	No
AP-2γ	Santa Cruz	Mouse	1:20	Yes
AMH	Santa Cruz	Goat	1:500	No
AR	Santa Cruz	Rabbit	1:200	Yes
c-KIT	Santa Cruz	Rabbit	1:50	No
HH3	Upstate Biotech	Rabbit	1:3000	Yes
Ki67(a)	DAKO	Mouse	1:40	Yes
Ki67(b)	Abcam	Rabbit	1:200	Yes
MAGE-A4	Gift[b]	Mouse	1:500	No
NANOG	R+D Systems	Goat	1:50	Yes
NANOS1	Abcam	Rabbit	1:500	Yes
OCT4(a)	Santa Cruz	Goat	1:50	Yes
OCT4(b)	Santa Cruz	Goat	1:200	Yes
Pan-cytokeratin	Sigma	Mouse	1:500	Yes
PCNA	DAKO	Mouse	1:100	Yes
PLAP	Abcam	Rabbit	1:100	Yes
SMA	Sigma	Mouse	1:5000	Yes
SOX9	Chemicon	Rabbit	1:80	Yes
VASA	Abcam	Rabbit	1:200	Yes

Table 2.1. Primary antibodies used for single immunohistochemistry.

[a] Prof. Ian Mason, The Queen's Medical Research Institute, Edinburgh, UK
[b] Dr. Guilio Spagoli, University Hospital, Basel, Switzerland

2.10.2.4. Secondary antibodies

The slides were washed twice more (5 minutes each) in TBS and incubated with a biotin conjugated secondary antibody (1:500), diluted in normal serum/TBS/BSA for 30 minutes at room temperature. Sections were washed again in TBS (5 minutes x2), followed by streptavidin-HRP (DAKO) diluted 1:1000 in TBS for 30 minutes. A summary of secondary antibodies used can be seen in Table 2.2.

2.10.2.5. Visualisation

3,3 Diaminobenzidine (DAB) was used as a substrate for the brown colour reaction to visualise the areas of the section to which the primary antibody had attached to the target protein. The DAB (DAKO) was diluted in DAB substrate buffer (1 drop/ml) and immediately applied to the sections for between 1 and 5 minutes, until the desired staining was achieved. Once adequate staining had occurred, the reaction was stopped by immersing the slides in tap water.

2.10.2.6. Haematoxylin counterstaining, dehydration and mounting

Sections were immersed in haematoxylin for between 4 and 6 minutes in order to lightly counterstain the nuclei of the cells. The sections were then immersed in tap water and placed into 1% acid alcohol for between 1 and 5 seconds and then rinsed again in tap water. The sections were then placed in Scott's tap water for 30 seconds to turn the nuclei blue and then rinsed again in tap water. The sections were then immersed for 20 seconds each in graded alcohols of increasing concentration (70%, 80%, 95%, absolute and absolute). The sections were then placed in xylene for 5 minutes and mounted under glass coverslips (VWR International) with Pertex mounting medium (CellPath Ltd, Hemel Hempstead, UK).

2.10.2.7. Imaging

Images were obtained using a Provis microscope (Olympus, London, UK) and photographed using a DCS3310 digital camera (Eastman Kodak, Rochester, NY). Images were assembled using Adobe Photoshop 7.0 (Adobe Systems Inc, Mountain View, CA, USA).

2.10.3. Double staining

In order to detect two different proteins in the same section, double staining was performed. This technique could be used for two primary antibodies that were raised in different species, thus allowing two different secondary antibodies to be used. Visualisation was performed with two different substrates producing

different coloured staining for each protein. The first steps were identical to those described for single staining (2.10.2.1 – 2.10.2.5). Once the sections were visualised with DAB the sections were washed twice (5 minutes each) in TBS and blocked in the appropriate normal serum/TBS/BSA for 30 minutes. The second primary antibody diluted in normal serum/TBS/BSA was then applied overnight at 4°C. The next day two 5 minute washes in TBS were followed by incubation with a biotin conjugated secondary antibody (1:500) diluted in normal serum/TBS/BSA for 30 minutes (Table 2.2). The sections were washed twice in TBS (5 minutes each) and incubated with streptavidin-alkaline phosphatase (DAKO) diluted 1:400 in TBS for 30 minutes. Two further TBS washes (5 minutes each) were followed by visualistion using fast blue as the substrate to produce a blue colour. The fast blue was prepared by adding 1mg of fast blue salt (Sigma) to 1ml of fast blue buffer. This solution was filtered through a 0.2μm filter (Millipore, Massachusetts, USA) to remove the undissolved salt. This solution was applied to the sections and kept in the dark for approximately 20 minutes until the desired staining was achieved. Counterstaining was not performed for double stained slides as the blue colour of the fast blue would be difficult to distinguish from the blue staining of the haematoxylin. The slides were mounted under glass coverslips with Permafluor (Beckman Coulter, High Wycombe, UK) mounting medium and images were obtained as for single staining (2.10.2.7). Details of double staining antibodies and detection methods are shown in Table 2.2.

Antigen 1	Dilution	Secondary antibody	Detection	Antigen 2	Dilution	Secondary antibody	Detection
OCT4(a)	1:50	RAG-b	DAB	MAGE-A4	1:20	RAM-b	Fast blue
VASA	1:200	SAR-b	Fast blue	OCT4(a)	1:50	RAG-b	DAB
Ki67(b)	1:200	SAR-b	DAB	MAGE-A4	1:10	RAM-b	Fast blue
Pan-cytokeratin	1:500	GAM-b	Fast blue	AR	1:200	GAR-b	DAB
Pan-cytokeratin	1:500	RAM-b	DAB	OCT4(b)	1:200	RAG-b	Fast blue
VASA	1:200	SAR-b	Fast blue	Ki67(a)	1:40	GAM-b	DAB
SOX9	1:80	SAR-b	Fast blue	OCT4(a)	1:20	RAG-b	DAB
OCT4(a)	1:50	DAG-b	Fast blue	c-KIT	1:20	GAR-b	DAB
c-KIT	1:20	GAR-b	DAB	MAGE-A4	1:20	RAM-b	Fast blue

Table 2.2. Antibodies and detection used for double immunohistochemistry

Abbreviations:
SAR-b – Swine anti-rabbit biotinylated (DAKO)
RAG-b – Rabbit anti-goat biotinylated (Vector Labs.)
RAM-b – Rabbit anti-mouse biotinylated (DAKO)
GAM-b – Goat anti-mouse biotinylated (DAKO)
GAR-b – Goat anti-rabbit biotinylated (DAKO)
DAG-b – Donkey anti-goat biotinylated (Abcam)

2.11. Immunofluorescence

Sections were prepared as described (2.8.1 – 2.8.3) and subjected to antigen retrieval and blocking as described for immunohistochemistry (2.10.2.1 – 2.10.2.3) TBS washes were replaced with phosphate buffered saline (PBS; Sigma).

2.11.1. Double immunofluorescence

This technique was used to visualise antibodies binding to two different proteins, particularly when these proteins are localised to the same regions of a particular cell type. The method used combinations of indirect immunofluorescence (section 2.10.1, using a fluorescent label) and the tyramide signal amplification (TSA) system (Fig. 2.6). TSA™ Plus (Perkin Elmer) involves addition of horseradish peroxidase (HRP) labelled secondary antibody. The HRP is used to catalyze the deposition and binding of a labeled tyramide adjacent to the immobilised HRP enzyme. This binding is covalent because the reaction intermediate dimerizes with tyrosine

residues on the surface-bound endogenous proteins. Since the added labels are only deposited proximal to the enzyme site, there is minimal, if any, loss of resolution.

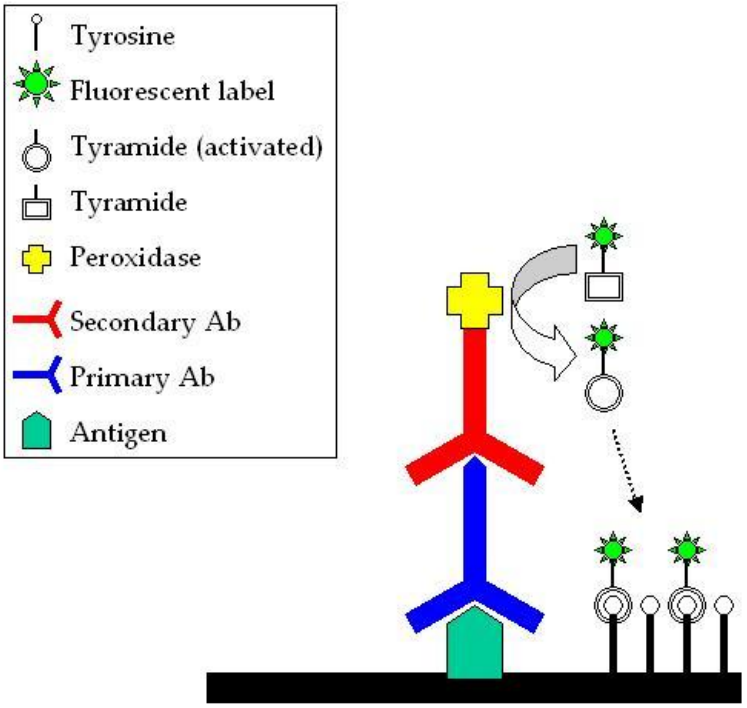

Figure 2.6. Tyramide signal amplification. HRP conjugated to the secondary antibody catalyses the activation of the tyramide. The activated tyramide bonds to the tyrosine residues on the tissue surface and the fluorescent label on the tyramide can be detected.

2.11.1.1. First primary antibody

Sections were incubated overnight with the first primary antibody diluted in normal serum/PBS/BSA (Table 2.3).

2.11.1.2. Secondary antibodies

The slides were washed twice more (5 minutes each) in PBS and incubated with a biotin or peroxidase conjugated secondary antibody (1:500), diluted in normal serum/PBS/BSA for 30 minutes at room temperature (Table 2.3). Sections were washed again in PBS (5 minutes x2).

2.11.1.3. Detection reagent for first primary antibody

The slides were kept in the dark for the remainder of the experiment to prevent loss of the fluorescent signal. For biotin conjugated secondary antibodies, slides were incubated with streptavidin conjugated to a fluorescent label that emits signal at a wavelength of 488 nm (Molecular Probes, Leiden, Netherlands), diluted 1:200 in PBS, for 60 minutes. For peroxidise conjugated secondary antibodies, slides were incubated with Tyramide Cy3 or Cy5, diluted 1:50 in the supplied buffer, for 10 minutes.

2.11.1.4. Blocking

The slides were then blocked with normal serum/PBS/BSA for 30 minutes, the choice of which depended on the source of the second primary antibody (Table 2.3)

2.11.1.5. Second primary antibody

Sections were incubated overnight with the second primary antibody in normal serum/PBS/BSA (Table 2.3).

2.11.1.6. Secondary antibodies

The slides were washed twice more (5 minutes each) in PBS and incubated with a biotin or peroxidise conjugated secondary antibody at a dilution of 1:500, diluted in normal serum/PBS/BSA for 30 minutes at room temperature (Table 2.3). Sections were washed again in PBS (5 minutes x3).

2.11.1.7. Detection reagents for second primary antibody

Slides that had been incubated with a biotinylated secondary antibody were incubated with streptavidin conjugated to a fluorescent label that emits signal at a wavelength of 546 nm and diluted 1:200 with PBS for 60 minutes. Alternatively for primary antibodies that required increased signal amplification a peroxidise labelled secondary antibody was used instead of the biotinylated secondary and a Tyramide labelled detection system was used. Slides were incubated for 10 minutes with Tyramide-Cy3 or Cy5 diluted 1:50 with the supplied buffer (Table 2.3).

2.11.1.8. Variation for detection of two primary antibodies raised in the same species

A variation was required for double staining involving primary antibodies that were raised in the same species (Brown et al., 2004). The secondary antibodies were both biotinylated FAb fragments (both 1:500, diluted in normal serum/PBS/BSA), which prevented cross-reaction between the two detection reagents. In addition a second streptavidin and biotin block (2.10.2.2) was required after the first detection. Detection reagents were strepatavidin-488 and streptavidin-546 (as described above) for first and second detection reagents respectively. Details of the antibodies used and the detection methods are shown in Table 2.3.

Antigen 1	Dilution	Secondary antibody	Detection	Antigen 1	Dilution	Secondary antibody	Detection
OCT4(a)	1:100	RAG-p	Tyr Cy3	AP-2γ	1:60	GAM-p	Tyr Cy5
PLAP	1:20	CAR-b	Strept 488	OCT4(a)	1:200	CAG-p	Tyr Cy3
NANOG	1:150	CAG-p	Tyr Cy5	OCT4(a)	1:150	CAG-p	Tyr Cy3
VASA	1:200	GAR-b	Strept 488	AP-2γ	1:60	GAM-p	Tyr Cy3
AP-2γ	1:60	GAM-p	Tyr Cy3	NANOS1	1:60	GAR-b	Strept 488
VASA	1:200	GAR-b	Strept 488	OCT4(a)	1:100	RAG-p	Tyr Cy3
VASA	1:200	GAR(fab)-b	Strept 488	NANOS1	1:60	GAR(fab)-b	Strept 546
AP-2γ	1:60	GAM-p	Tyr Cy3	Ki67(b)	1:100	GAR-b	Strept 488
NANOG	1:150	CAG-p	Tyr Cy5	VASA	1:100	CAR-p	Tyr Cy3

Table 2.3. Antibodies and detection used for double immunofluorescence

Abbreviations:
RAG-p – Rabbit anti-goat peroxidase (Sigma)
GAM-p – Goat anti-mouse peroxidase (Santa Cruz)
CAR-p – Chicken anti-rabbit peroxidase (Santa Cruz)
CAR-b – Chicken anti-rabbit biotinylated (Santa Cruz)
CAG-p – Chicken anti-goat peroxidase (Santa Cruz)
GAR-b – Goat anti-rabbit biotinylated (Vector)
Tyr Cy3 – Tyramide Cy3 (Perkin Elmer)
Tyr Cy5 – Tyramide Cy5 (Perkin Elmer)
Strept 488 – Streptavidin 488 (Molecular probes)
Strept 546 – Streptavidin 488 (Molecular probes)

2.11.1.9. Counterstaining, mounting and imaging

TO-PRO-3 (Molecular Probes, Leiden, Netherlands) was applied at 1:1000 in PBS for 5 min as a nuclear counterstain and the slides were mounted using Permafluor.

Images were captured using an LSM 510 Confocal microscope (Carl Zeiss, Hertfordshire, UK).

2.11.2. Triple immunofluorescence

For triple immunofluorescence, further periods in boiling citrate between each primary antibody was required to prevent cross reaction. TSA™, plus enhanced detection systems were required for each primary antibody as the streptavidin based fluorescent antibodies were unable to withstand repeated boiling in citrate. Sections were prepared as described (2.8.1 – 2.8.3) and subjected to antigen retrieval and blocking as described for immunohistochemistry (2.10.2.1 – 2.10.2.3) with PBS washes replacing TBS.

2.11.2.1. First primary antibody

After blocking with normal serum/PBS/BSA for 30 minutes, sections were incubated overnight with the first primary antibody in normal serum/PBS/BSA.

2.11.2.2. Secondary antibodies

The slides were washed twice more (5 minutes each) in PBS and incubated with a peroxidase conjugated secondary antibody (Table 2.4), diluted in normal serum/PBS/BSA for 30 minutes at room temperature. Sections were washed again in PBS (5 minutes x2).

2.11.2.3. Detection reagent for first primary antibody

The slides were kept in the dark for the remainder of the experiment to prevent loss of the fluorescent signal. Slides were incubated with labelled Tyramide diluted in buffer at 1:50 for 10 minutes.

2.11.2.4. Blocking cross reactivity

Sections were placed in a plastic rack and into a glass trough containing citrate buffer and covered in pierced cling film. The slides were then microwaved on full

power for 2.5 minutes using a conventional microwave oven and then left in the hot citrate for 30 minutes. The slides were then washed in PBS for 5 minutes.

2.11.2.5. Second and third primary antibodies

The two remaining primary antibodies were incubated sequentially by repeating steps described in section 2.11.2.1 – 2.11.2.4 for the second antibody and 2.11.2.1 – 2.11.2.3 for the final antibody, using detection with Tyramide conjugated to different fluorescent labels.

2.11.2.6. Counterstaining, mounting and imaging

DAPI (Sigma) was applied at 1:1000 in PBS for 10 min as a nuclear counterstain and the slides were mounted using Permafluor. Images were captured using an LSM 510 Meta Confocal microscope (Carl Zeiss). Details of the antibodies used and the detection methods are shown in Table 2.4

	VASA PLAP OCT4	**MAGE-A4 OCT4 PLAP**	**OCT4 MAGE-A4 Ki67**
Antigen 1	VASA	MAGE-A4	OCT4(a)
Dilution	1:1000	1:100	1:150
Secondary antibody	GAR-(fab)-p	CAM-p	CAG-p
Detection	Tyr Cy5	Tyr Cy5	Tyr Cy3
Antigen 2	PLAP	OCT4(a)	MAGE-A4
Dilution	1:200	1:150	1:100
Secondary antibody	GAR-(fab)-p	CAG-p	CAM-p
Detection	Tyr Fluorescein	Tyr Cy3	Tyr Cy5
Antigen 3	OCT4(a)	PLAP	Ki67(a)
Dilution	1:150	1:200	1:150
Secondary antibody	CAG-p	CAR-p	CAM-p
Detection	Tyr Cy3	Tyr Fluorescein	Tyr Fluorescein

Table 2.4. Antibodies and detection used for triple immunofluorescence.

Abbreviations:
GAR-(fab)-p – Goat anti-rabbit-FAB-peroxidise (Abcam)
CAM-p – Chicken anti-mouse peroxidase (Sigma)
CAR-p – Chicken anti-rabbit peroxidase(Sigma)
CAG-p – Chicken anti-goat peroxidase (Sigma)
Tyr Cy3 – Tyramide Cy3 (Perkin Elmer)
Tyr Cy5 – Tyramide Cy5 (Perkin Elmer)
Tyr Fluorescein – Tyramide Fluorescien (Perkin Elmer)

2.12. Image analysis

2.12.1. Image analysis - immunohistochemistry

Quantification of germ cells and proliferation counts on sections obtained from immunohistochemistry were performed using Image-pro Plus 4.5 software (Media Cybernetics, Wokingham, Berkshire, UK). Live images were obtained using a Zeiss microscope (Carl Zeiss) fitted with an automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Sections were tiled using a 40x objective and an area of interest (AOI) was drawn around the entire section. Random fields were generated and the total number of germ cells was calculated for each field based either on morphology and position or alternatively following staining with a germ cell marker. The total number of proliferating germ cells was also calculated.

2.12.2. Image analysis – immunofluorescence

2.12.2.1. Double immunofluorescence

In order to investigate whether GnRHa treatment of neonatal marmosets resulted in persistence of gonocytes (section 2.5.1) in 6 week old animals, the proportion of germ cells expressing NANOG was compared to the proportion in control co-twins. Quantification was performed using Image-pro Plus 4.5 software (Media Cybernetics, Wokingham, Berkshire, UK). Live images were obtained using a Zeiss microscope fitted with fluorescent filters (Zeiss) and an automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Sections were double stained with a general germ cell marker (VASA) in addition to the gonocyte maker of interest (Table 2.3). Sections were tiled using a 40x objective and an area of interest (AOI) was drawn around the section. The stage was moved around the entire section and the total number of germ cells (VASA positive) in the entire section was counted using the red filter. Once all the germ cells had been counted the stage was again moved across the whole section using the green filter and the total number of cells expressing the gonocyte marker (NANOG positive) was calculated. The number of cells expressing the gonocyte marker was then divided by the total number of germ

cells to give the proportion of germ cells expressing the gonocyte marker. Data from each treated animal was compared to its co-twin and statistical analysis was performed with a students paired t-test using Graphpad Prism 5 software (La Jolla, California USA). A p-value of <0.05 was considered significant.

2.12.2.2. Triple immunofluorescence

Quantification of germ cell subpopulations and proliferation indices were performed using Axiovision 4.6 software (Carl Zeiss) and images obtained using an Axiovert 200M microscope with attached Axiocam HRc camera and an MCU2P automatic stage (all Carl Zeiss). Fluorescent filters were attached to detect green, blue and red fluorescence. The multidimensional acquisition function was used and the appropriate channels selected. The exposure of each of the fluorochromes was measured individually. The 'mark and find' function was then used to acquire a background image. Using the MosaiX function at a magnification of 5x a tiled image was created with the DAPI filter on. This allowed the observer to identify the boundaries of the sample and identify the areas of interest without being able to see the individual staining. Points within the desired area were randomly chosen (Fig. 2.7) and the focus (including z co-ordinate) for each position adjusted manually.

For each field, an image was captured for every individual channel, in addition to a merged image. These images were then exported as JPEG files for quantitative analysis. Quantitative analysis was performed using Adobe Photoshop 7.0 (Adobe, San Jose, USA). All of the images for an individual field were opened (Fig. 2.8).

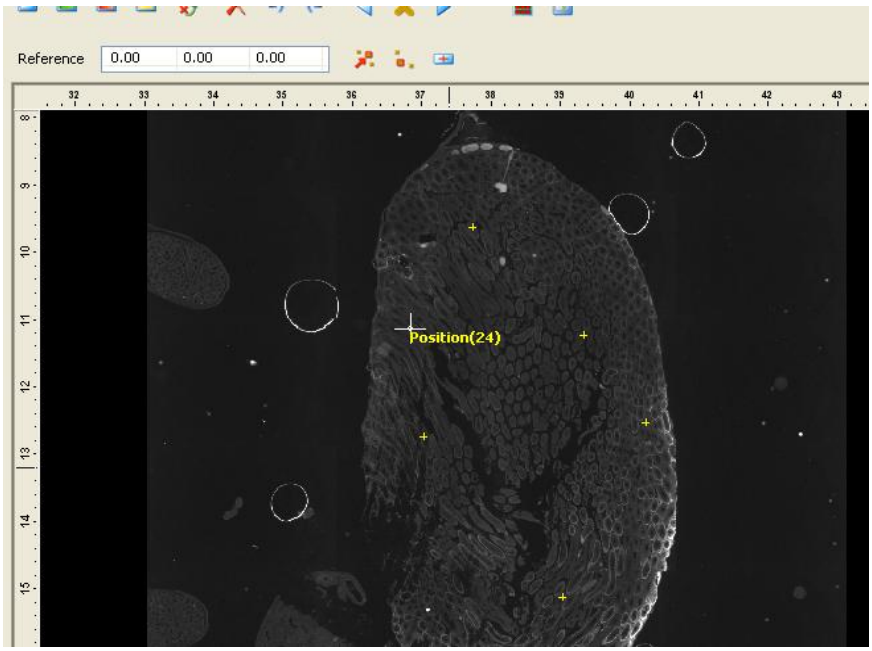

Figure 2.7. Generating random points for quantification of cell types within triple stained sections. Random points are generated within the tiled image using the DAPI filter.

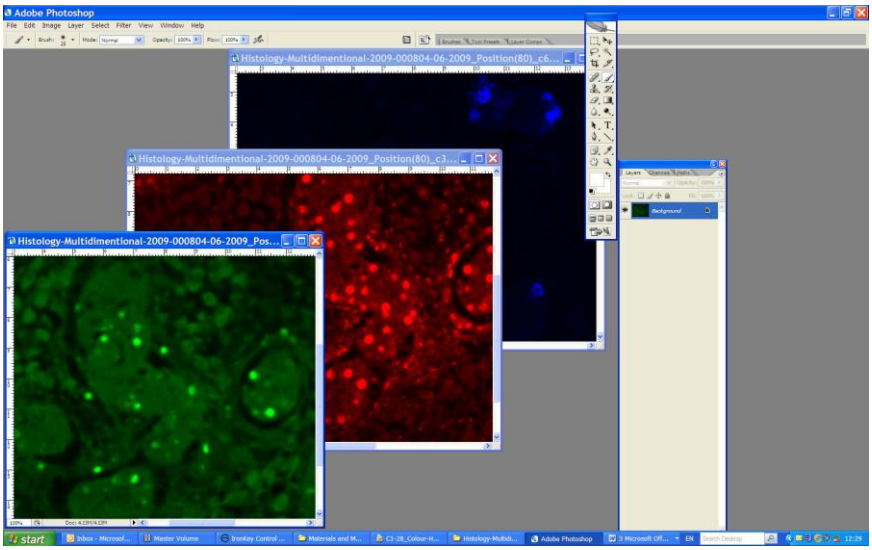

Figure 2.8. Opening images in Photoshop for counting cells from triple staining experiments.

The images were kept at a constant size and the cells within the first image marked and counted by creating a new layer and using the paint function to mark the cells (Fig. 2.9)

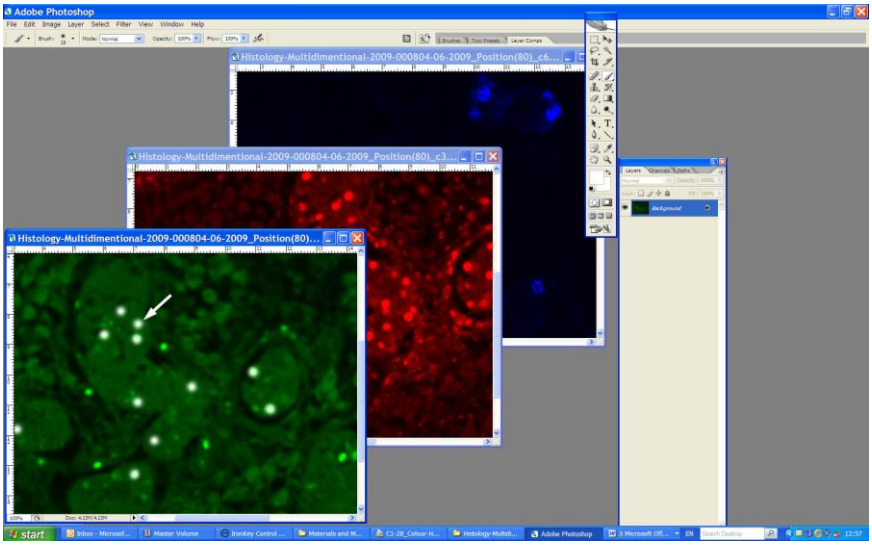

Figure 2.9. Marking the first layer of stained cells. Cells labelled with the green tyramide signal were marked in white (arrow) using the paint function.

This layer was then duplicated onto the next image (Figure 2.10). A new layer was then created and the co-stained cells were marked with the paint function using a different colour to the first mark. The remaining single stained cells could then be marked on a further layer. In order to visualise the cells beneath the superimposed layers, the hide layers function could be turned on and off as the cells were counted. The cells were counted manually. This process could be repeated to duplicate marks onto a third layer.

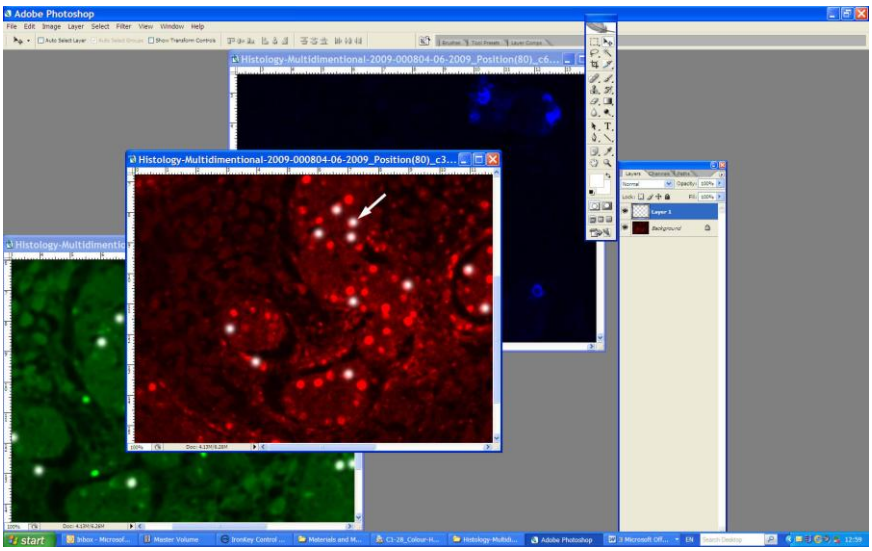

Figure 2.10. Duplication of the paint marks from one image onto the subsequent layer.

The white paint marks (arrow) are duplicated from the green image to the red image.

Using this technique it was possible to count all the combinations of single, double and triple stained cells. In addition, the presence of nuclei could be established by superimposing layers onto the DAPI image in order to confirm the presence of a nucleus in situations where a cell was negative for staining with a nuclear marker and thus rule out 'false negatives'. Statistical analysis was performed with a students t-test using Graphpad Prism 5 software. A p-value of <0.05 was considered significant.

2.13. Western immunoblotting

2.13.1. Protein extraction and quantification

Frozen testis tissue from human fetuses and marmoset (neonatal to adult), were used for protein extraction. Fat and ductal tissues were removed from the testis and the tissue was cut into small pieces and placed into Eppendorf tubes containing 200µl RIPA buffer. Samples were then homogenised and incubated on ice for 1 hour before being centrifuged (2500rpm) for 10 minutes at 4 ^{o}C. The supernatant was removed.

Protein quantification was performed using the Biorad assay (Bio-Rad, California, US) to compare the protein content of the samples to those of known concentrations of BSA. Standards of BSA in RIPA buffer were prepared in concentrations ranging from 0.125mg/ml to 1.5mg/ml. 20µl of Solution S was added to 1ml Buffer A. Aliquots of 25µl of this solution were then added to the wells of a clear 96 well plate. To these wells, 5µl aliquots of the protein/standard were added in duplicate. Finally 200µl of Reagent B was added to the wells. The samples were left to incubate for 15 minutes at room temperature. A blue colour was produced by the reduction of the folin reagent by the protein, which had initially reacted with copper. The intensity of the colour was determined using a Labsystems Multiskan EX plate reader (VWR) at 698nm. A standard curve was generated from the readings for the standards using Microsoft Excel and the volume required to obtain 10µg protein was calculated.

2.13.2. Gel Electrophoresis and Transfer

Samples were prepared by adding 2.5µl 4x NuPAGE LDS Sample Buffer to 1µl of 10x NuPAGE Reducing Agent (both Invitrogen, California, US). The appropriate volume of sample for 10µl protein was then added and each vial was made up to a total of 10µl with deionised water. The samples were then heated at 70°C for 10 minutes before being transferred to ice. A 10% NuPAGE Bis-Tris gel (Invitrogen) was loaded into a Novex Mini-cell gel tank (Invitrogen) containing 1x NuPAGE MOPS SDS running buffer (Invitrogen) and samples were loaded along with 5µl SeeBlue Plus2 pre-stained standard (Invitrogen), in a parallel lane. The gel was run at 200V for 50 minutes. The gel was removed from the tank and transferred to a nitrocellulose imobilin FP membrane (Millipore) pre-soaked in methanol. Successive layers of scourers x2, Whatman 3MM paper x3 (Whatman, Brentford, UK), gel, membrane, Whatman paper x3, scourers x2 were loaded into a cassette and placed into a Hoefer TE-22 transfer tank (Amersham Biosciences, San Fransisco, US) filled with 1x transfer buffer (Invitrogen) and run at 20V overnight.

2.13.3. Immunoblotting

The membrane was removed and washed twice in PBS for 5 minutes each. The membrane was then blocked in Odyssey blocking buffer (LI-COR Biosciences, Cambridge, UK): PBS (1:1), for 1 hour. The membrane was incubated with the primary antibody (Table 2.5) and a loading control (β-tubulin) diluted in blocking buffer/PBS containing 0.1% Tween, for 1 hour. This was followed by four washes in PBS Tween (0.1%) for 5 minutes each. The membranes were then incubated with secondary antibodies diluted 1:10000 in Odyssey blocking buffer/PBS containing 0.1% Tween, for 1 hour. Details of the secondary antibodies can be seen in Table 2.5. A further 4 washes in PBS Tween (0.1%) for five minutes each followed before fluorescent images were obtained. Images were scanned using the Licor Odyssey system and software (LI-COR Biosciences).

	Primary antibody	**Species**	**Source**	**Dilution**	**Secondary antibody**	**Source**	**Detection Wavelenghth**
1	VASA	Rabbit	Abcam	1:500	Goat anti-rabbit	Rockland USA	800nm
	β-tubulin	Mouse	Sigma	1:1000	Goat anti-mouse	Molecular Probes	680nm
2	PLAP	Rabbit	Abcam	1:500	Goat anti-rabbit	Rockland USA	800nm
	β-tubulin	Mouse	Sigma	1:1000	Goat anti-mouse	Molecular Probes	680nm

Table 2.5. Antibodies and detection used for Western blotting.

2.14. Commonly Used solutions

Citrate buffer

- 42.02g Citric acid monohydrate
- 1900ml Distilled H_2O
- Adjust pH to 6.0 with NaOH

Fast Blue Buffer

- 12.1g Tris
- 950ml Distilled H_2O
- Make up to 1L at pH 8.2 with HCl
- Take 98ml of solution from above, add
- 20mg Napthol AS-MX phosphate
- 2ml Dimethyl formamide

Scott's Tap water

- 10g Potassium chloride
- 100g Magnesium sulphate
- Tap water 5L

RIPA buffer

- 25mM tris
- 1% triton
- 0.05% sodium deoxycholate
- 0.1% SDS
- 150mM NaCl

Tris-Acetate (TE) Buffer

- 242g Tris base
- 57.1ml Acetic acid

- 100ml 0.5M EDTA
- Adjust pH to 8.5

Tris-buffered Saline (TBS)

- 60.5g Tris base
- 87.6g NaCl
- 300mls HCl
- Adjust pH to 7.4 with HCl

3 The origins of CIS and TGCT in the human testis

3.1. Introduction

3.1.1. Carcinoma in situ as the precursor lesion for TGCT

It has been known for some years that the precursor cells that ultimately lead to a TGCT are the CIS cells (Skakkebaek, 1972b). This hypothesis is supported by the temporal relationship between CIS and TGCT in addition to the shared features between the two (1.21.3). CIS is present in biopsies of the testis of patients who subsequently develop a TGCT and eventually all patients with CIS will develop a TGCT (Oosterhuis and Looijenga, 2005).

3.1.2. The origin of CIS cells from fetal germ cells

CIS cells are thought to be present in the testis from fetal life and have been hypothesised to arise from abnormally differentiated gonocytes (Skakkebaek et al., 1987). This is supported by the fact that CIS cells have a similar morphological appearance to fetal gonocytes. Both cells are relatively large compared to spermatogonia and have abundant clear cytoplasm. CIS cells also express of a variety of proteins that are involved in pluripotency and early germ cell fate, such as PLAP (Jorgensen et al., 1995);(Hustin et al., 1987) and OCT4 (Jones et al., 2004);(Rajpert-De Meyts et al., 2004);(Looijenga et al., 2003a). Germ cell specific proteins that are expressed in germ cells during fetal life, such as VASA are also expressed in CIS cells (Castrillon et al., 2000);(Zeeman et al., 2002). The precise period for germ cells to transform into CIS cells remains uncertain and indeed may occur at different stages of fetal germ cell development (Rajpert-De Meyts et al., 1998).

3.1.3. Heterogeneity of CIS cells

It is reported that all CIS cells express OCT4 (Looijenga et al., 2003a). Expression persists in seminoma and embryonal carcinoma before being downregulated upon

differentiation of embryonal carcinoma to a non-seminoma (Oosterhuis and Looijenga, 2005). Other proteins are expressed in CIS cells, however it has been noted that there exists a heterogeneity of expression of many of these proteins. Whilst PLAP is described as being present in 83-99% of CIS cells, other proteins (TRA-1-60, VASA and MAGE-A4) are expressed in a more heterogeneous manner (Rajpert-De Meyts, 2006). Despite the knowledge that this heterogeneity exists, the relationship between cells of different phenotypes and their malignant and proliferative potential has not been extensively investigated.

3.1.4. Proliferation in CIS cells

CIS cells have a high rate of mitotic divisions (Skakkebaek, 1972b). The majority of sections from patients with TGCT contain CIS cells that express Ki67 and this is the case for both seminoma and non-seminoma (Datta et al., 2000), however no indication of other phenotypic characteristics of the Ki67 positive CIS cells have been described.

3.1.5. Sertoli cell development and relationship to CIS development

A close relationship exists between the Sertoli cell and the germ cells within the normal testis (1.19). Studies have investigated the relationship between CIS cells and their associated Sertoli cells within the testes of patients with TGCT (Brehm and Steger, 2005);(Brehm et al., 2006);(Rajpert-De Meyts and Skakkebaek, 1992). Cytokeratin 18 (Ck18) is expressed by the Sertoli cells of the human fetal testis and downregulated in early postnatal life and is not expressed in the normal adult testis (Brehm et al., 2006);(Stosiek et al., 1990). In adults, expression of Ck18 has been described in the tubules containing CIS (Brehm and Steger, 2005). AMH is expressed in the seminiferous tubules of the testis from fetal life until puberty, when it is downregulated (Brehm et al., 2006). Androgen receptor expression is present in the Sertoli cells in the tubules of normal adult testes (Brehm et al., 2006) and CIS containing tubules (Rajpert-De Meyts and Skakkebaek, 1992). The relationship

between the differentiation status of the Sertoli cells and the phenotype of the associated CIS cells remains to be established.

3.2. Chapter aims

This chapter aimed to further characterise the CIS cell in terms of expression of common markers of fetal germ cells, and to identify subpopulations of CIS cells that may provide insight into the cell of origin. An additional aim was to investigate the relationship between these CIS subpopulations and their proliferation status, as well as the functional development of their associated Sertoli cells.

3.3. Materials and methods

3.3.1. Human testicular germ cell tumour tissue

Tissue from patients with testicular germ cell tumours was obtained as described in section 2.1.2. A minimum of three pre-invasive CIS, seminoma and teratoma samples were investigated for each experiment.

3.3.2. Human fetal testis tissue collection

Human fetal testis tissue for use in immunohistochemical studies and Western blots were obtained as described in Section 2.4.2. The sections used were from 14 - 19 week gestation fetuses.

3.3.3. Haematoxylin and eosin staining

The method for haematoxylin and eosin staining is described in section 2.9

3.3.4. Immunohistochemistry

Single immunohistochemistry was performed with DAB detection as described in section 2.10.2. The primary antibodies used for these experiments are listed in Table 2.1. Double staining was performed with DAB and fast blue detection as outlined in section 2.10.3. Details of conditions for double staining experiments can be found in Table 2.2.

3.3.5. Immunofluorescence

Double immunofluorescence was performed as described in section 2.11.1. The primary and secondary antibodies, and detection labels used for these experiments are listed in Table 2.3. Triple immunofluorescence was performed as described in section 2.11.2. The primary and secondary antibodies, and detection labels used for these experiments are listed in Table 2.4.

3.3.6. Quantification of CIS subpopulations and proliferation

Quantification of subpopulations of CIS cells in double stained cells is described in section 2.12.1. Sections of pre-invasive CIS, seminoma and teratoma were randomly chosen (n=3). The proportion of germ cells expressing the various combinations of markers was calculated by dividing the number of CIS cells with an individual expression profile by the total CIS cells, for a minimum of 40 random fields. Statistical analysis was performed using a one-way analysis of variance (ANOVA).

Quantification of triple stained immunofluorescent sections was performed as described in section 2.12.2.2. Sections of pre-invasive CIS, seminoma and teratoma were randomly chosen (n=3). The proportion of germ cells expressing the different combinations of markers was calculated as described above and a proliferation index for each subpopulation was calculated by dividing the number of proliferating (Ki67 positive) cells by the total number of cells within that subpopulation. Statistical analysis was performed using the unpaired students t test.

3.3.7. Western blotting

Fetal testis tissue was obtained for Western analysis as described in section 2.13.1. Details of the protein extraction, quantification and Western blotting procedure can be found in section 2.13. Details of the primary antibodies used are found in Table 2.5.

3.4. Results

3.4.1. Histology of seminiferous tubules containing CIS and normal spermatogenesis in adults with TGCT

Patients with TGCT display a variety of tubule types within the testis (Fig. 3.1). Some areas of the affected testis may contain tubules that have active spermatogenesis and appear histologically normal, whilst other tubules will contain the tumour precursor CIS cells. These cells have a characteristic appearance and line the basement membrane of the tubules. They contain a large amount of clear glycogen rich cytoplasm. These CIS cells can develop into either a seminoma or a non-seminoma (including teratoma) and some are mixed tumours containing both types within the same testis of an individual patient.

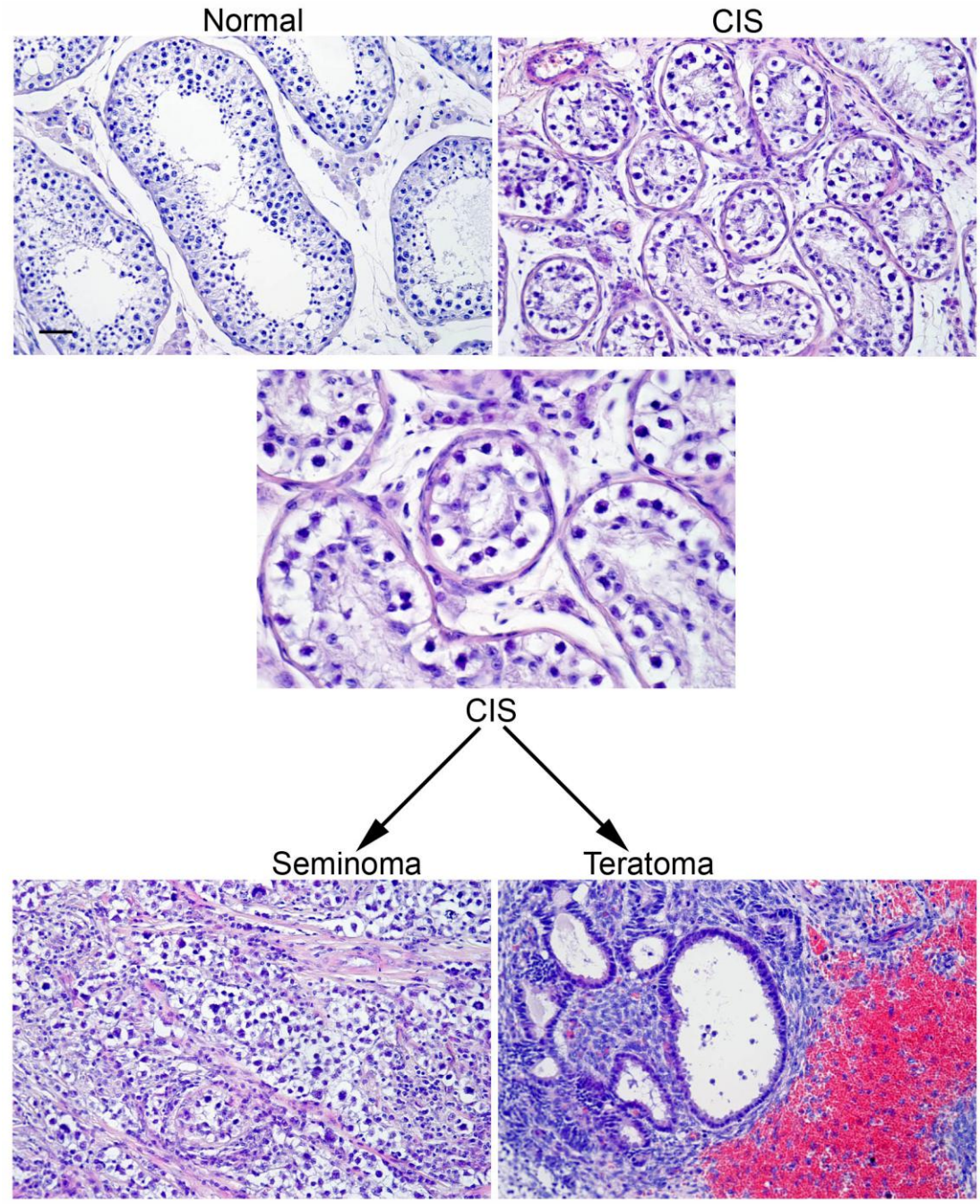

Figure 3.1. H+E. Sections taken from adult patients with TGCT. The affected testis may contain tubules with normal appearance of spermatogenesis and tubules with CIS (upper panels). CIS cells may develop into malignant tumours of either the seminoma (lower left) or non-seminoma (e.g. teratoma, lower right) types. Scale bar - 50μm.

3.4.2. Expression of pluripotency and PGC proteins in CIS and human fetal testis

Consistent with reports in the literature, we were able to confirm expression of proteins associated with pluripotency and undifferentiated germ cells in fetal germ cells are also expressed in CIS cells. These include OCT4, NANOG, AP-2γ and PLAP (Fig. 3.2).

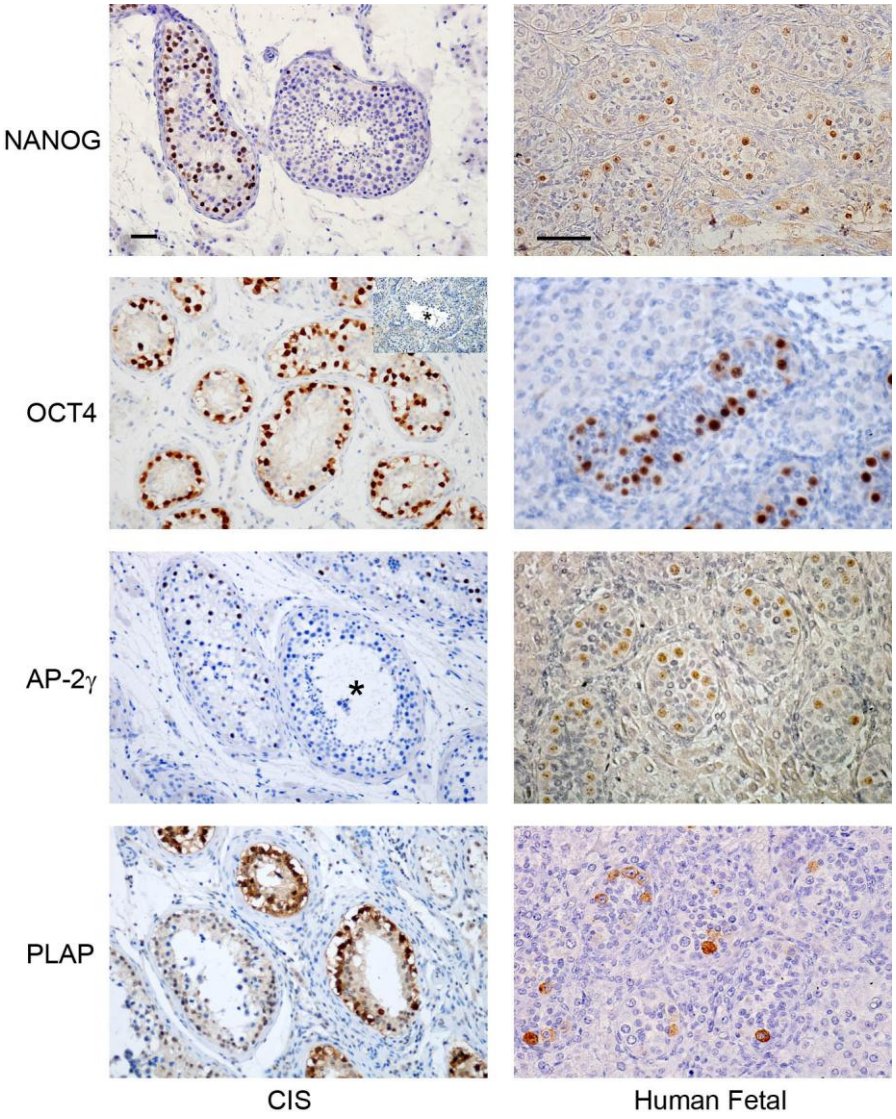

Figure 3.2. Expression of OCT4, NANOG, AP-2γ and PLAP in tubules containing CIS, from adult patients with TGCT. The same proteins can be identified in sections from TGCT (left panels) as are found in human fetal testes (right panels). In tubules with normal spermatogenesis there is no expression of these proteins (*). Scale bar - 50μm.

3.4.3. Expression of differentiating germ cell proteins in CIS and normal testis

In patients with a TGCT, VASA, MAGE-A4 and NANOS-1 are expressed in a proportion of cells within a CIS containing tubule. However there are cells within these tubules that have the phenotypic appearance of CIS cells, which are negative for these markers (Fig. 3.3). These proteins are also expressed in germ cells of tubules that contain active spermatogenesis in patients with a TGCT (Fig. 3.3).

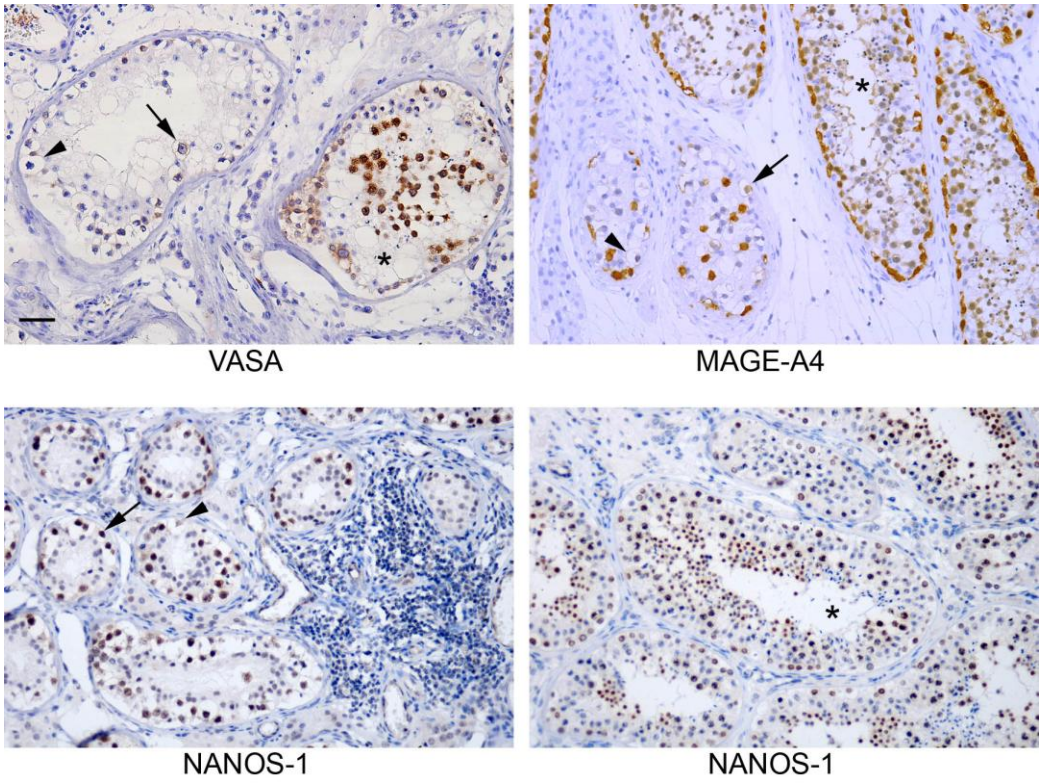

Figure 3.3. Expression of VASA, MAGE-A4 and NANOS-1 in CIS containing tubules from adult patients with TGCT. Cells resembling CIS cells can be identified that are positive (arrows) or negative (arrowheads) for these proteins. In tubules with active spermatogenesis there is expression of these proteins in normal germ cells (*). Scale bar - 50μm.

The antibodies for VASA and PLAP were tested on tissue from fetal human testes in order to show their specificity for the protein of interest. For VASA a single band of 67 KDa was obtained. PLAP was detected as a band at 58 KDa (Fig. 3.4).

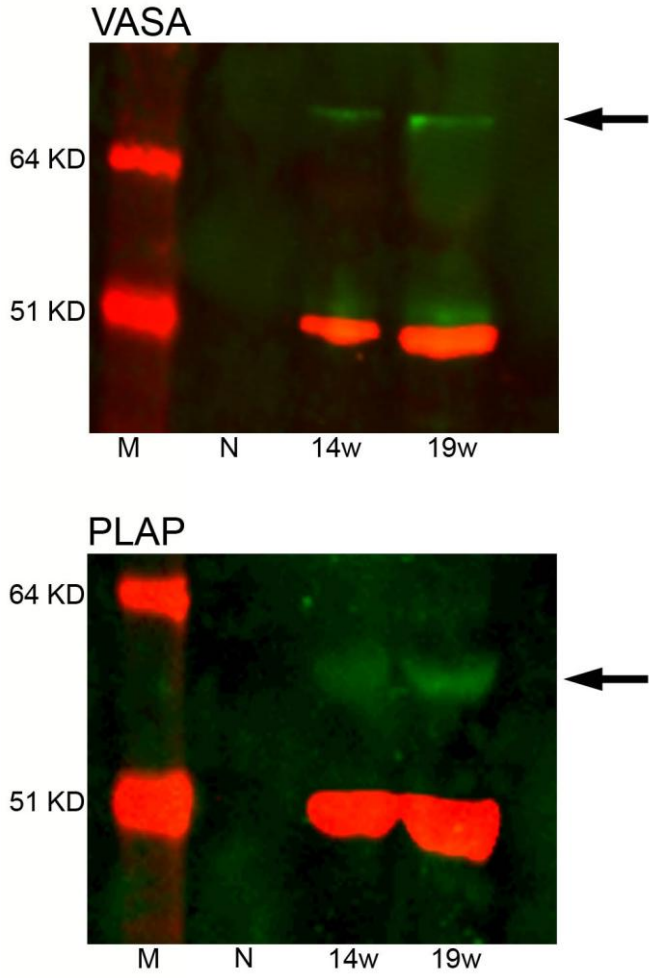

Figure 3.4. Western blots for VASA and PLAP in human fetal testes. β-tubulin (51 KDa) was used as a loading control. Black arrows represent bands at the expected MW for the expected proteins. M = molecular weight marker, N = negative, w = weeks.

3.4.4. Co-expression of early germ cell markers in tubules containing CIS cells

Fluorescent immunohistochemistry revealed that OCT4 and AP-2γ were co-expressed in the nuclei of the majority of germ cells in CIS containing tubules (Fig. 3.5a). NANOG and OCT4 were also expressed in the nucleus of the majority of germ cells within CIS containing tubules (Fig. 3.5b), although a few germ cells were $OCT4^{+}/NANOG^{-}$, whilst there were also occasional cells that were $OCT4^{-}/NANOG^{+}$. A similar pattern of expression was demonstrated by double staining of OCT4 and PLAP (Fig. 3.5c). In the CIS containing tubules most of the germ cells co-expressed

OCT4 and PLAP. There were also some germ cells that were $OCT4^{+}/PLAP^{-}$. These two populations can also be identified in the germ cells of the human fetal testis (Fig. 3.5d). However there were germ cells that were $OCT4^{-}/PLAP^{+}$ (Fig. 3.5c) in the CIS containing tubules, which were not identified in the human fetal germ cells (Fig. 3.5d).

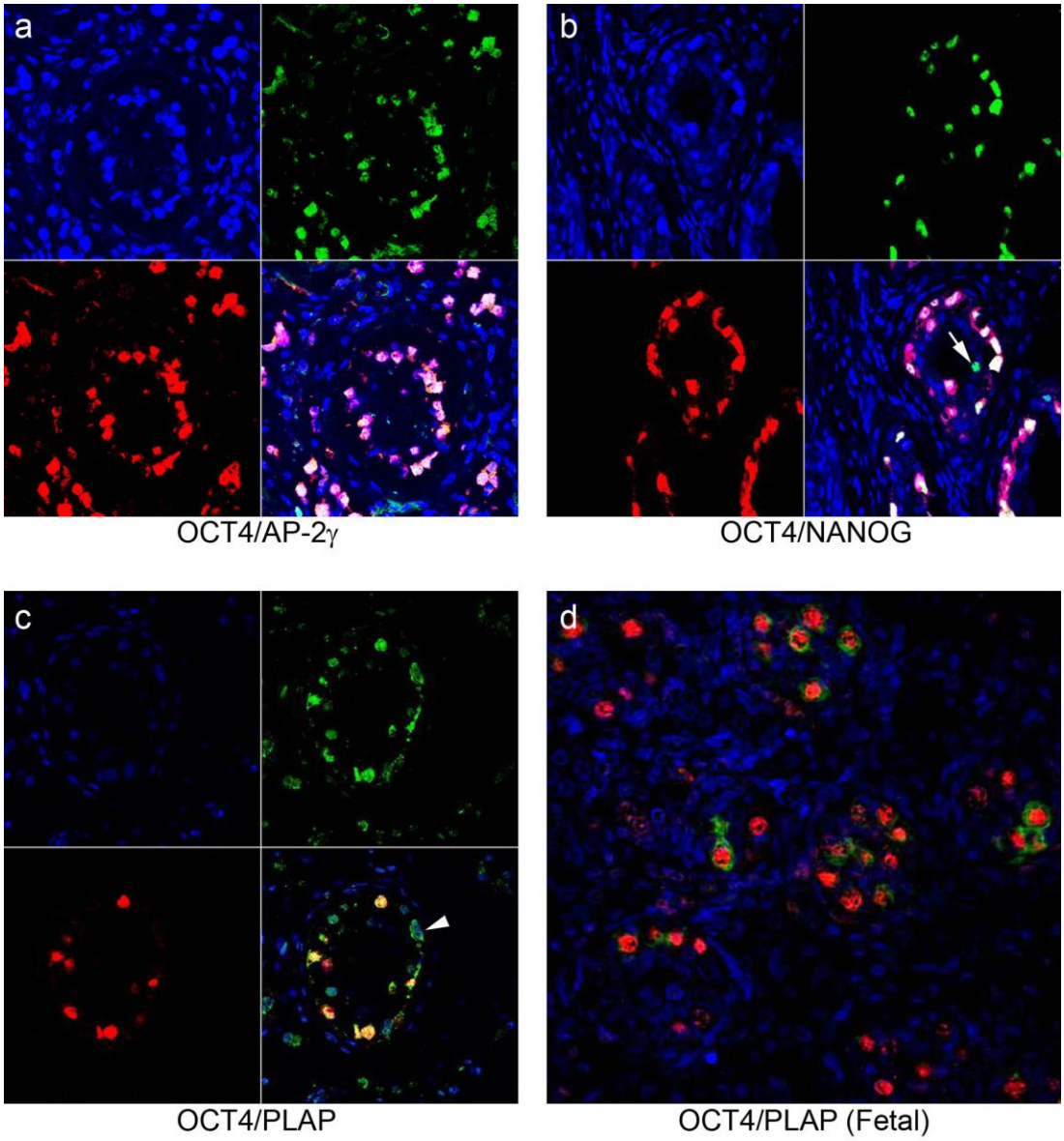

Figure 3.5. Co-expression of classical markers of CIS cells and gonocytes. OCT4 (red), AP-2γ (a; green), NANOG (b; green) and PLAP (c; green) in CIS containing tubules from adult patients with TGCT. Most putative CIS cells express OCT4, but occasional cells are $OCT4^{-}/NANOG^{+}$ (b; arrow) or $OCT4^{-}/PLAP^{+}$ (c; arrowhead). (d) Co-expression of OCT4 and PLAP in a 14 week human fetal testis. Note that $OCT4^{-}/PLAP^{+}$ cells are not present.

3.4.5. Identification of CIS cells based on the expression of germ cell proteins

In an attempt to distinguish CIS cells from normal germ cells, double staining for 'classical' CIS proteins (OCT4, AP-2γ, PLAP and NANOG) with proteins considered characteristic of differentiating germ cells (VASA, MAGE-A4 and NANOS-1) was undertaken. Tubules containing CIS cells had germ cells with variable phenotypes (Fig. 3.6). The majority of the germ cells expressed markers such as OCT4, which would be consistent with a CIS phenotype. Some of the cells co-expressed OCT4 with either VASA or MAGE-A4. The fact that they expressed OCT4 suggests that these are also CIS cells. However the majority of the cells that expressed VASA or MAGE-A4 were immunonegative for OCT4, which suggests that they may not be CIS cells but may be 'normal' germ cells within CIS containing tubules.

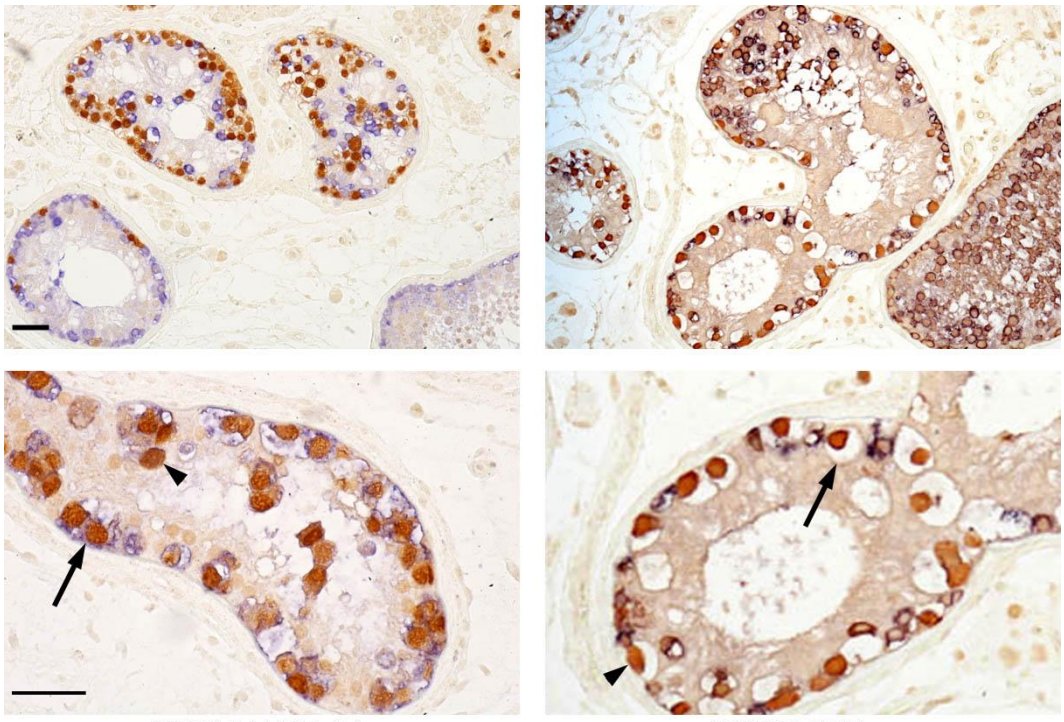

Figure 3.6. Co-expression of OCT4 and the germ cell specific proteins VASA and MAGE-A4 in sections from adult patients with TGCT. OCT4 (brown) and VASA or MAGE-A4 (both blue) were expressed in germ cells within CIS containing tubules. Cells resembling CIS cells can be identified that are positive for OCT4 alone (arrowheads) or both markers (arrows). Note the variation in expression patterns between tubules. Magnification x20 (upper panels) and x40 (lower panels). Scale bar - 50μm.

A similar expression pattern could be seen with double fluorescent staining of AP-2γ and VASA (Fig. 3.7). In tubules with a complement of normal germ cells, expression of VASA was detected but no AP-2γ positive cells were present (Fig. 3.7a). In tubules containing a mixture of germ cells with either CIS or normal phenotypes, the putative CIS cells (located on the basement membrane) expressed AP-2γ and a small proportion of these cells also co-expressed VASA (Fig. 3.7b). Putative 'normal' germ cells (located nearer the lumen) expressed VASA only and were immunonegative for AP-2γ. Different populations of germ cells as determined by their patterns of expression of AP-2γ, OCT4 and VASA were also identified within the tubules of the normal human fetal testis.

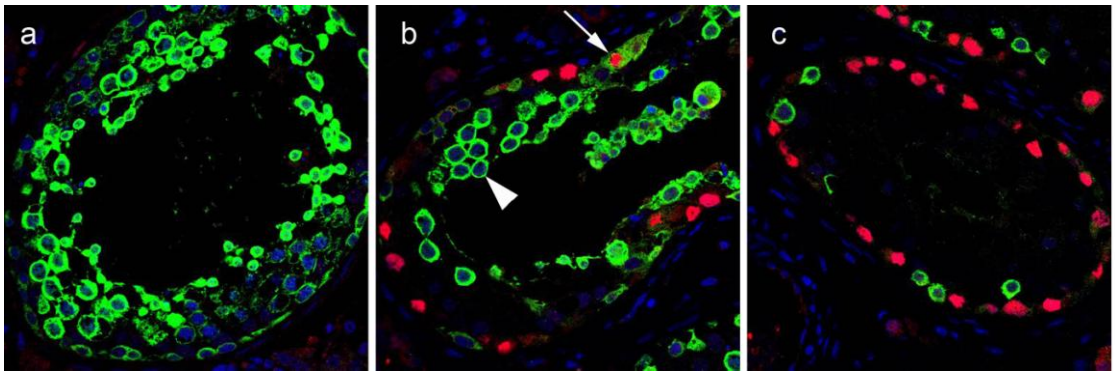

Figure 3.7. Expression of AP-2γ and VASA in CIS containing tubules from adult patients with TGCT. a) Tubule with normal spermatogenesis contains cells expressing VASA (green) only. b) Tubule with a mixture of cells expressing AP-2γ (red), AP-2γ/VASA (arrow, putative CIS cells) or VASA (arrowhead, putative 'normal' germ cells). c) Tubule with typical appearance of CIS with AP-2γ, (putative CIS cells) or VASA (putative 'normal' germ cells) located along the basement membrane. Sections counterstained with TO-PRO-3 (blue).

In the current study, NANOS-1 was detected in germ cells in tubules with active spermatogenesis, whilst AP-2γ was not present in these cells (Fig. 3.8a). In tubules containing CIS cells, NANOS-1 and AP-2γ were expressed in separate populations of germ cells with no co-localisation (Fig. 3.8b,c). This is in contrast to the results obtained when sections were co-stained for AP-2γ and VASA when both markers were co-localised in some germ cells (Fig. 3.7).

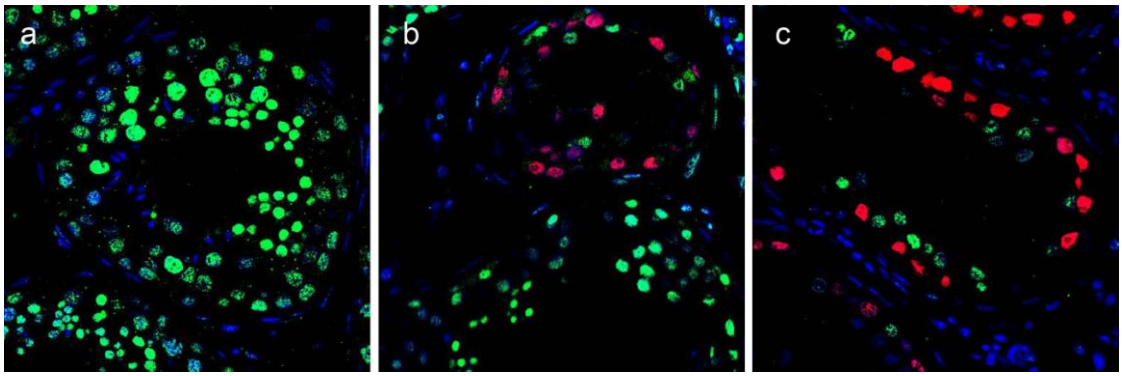

Figure 3.8. Expression of AP-2γ and NANOS-1 in CIS containing tubules from adult patients with TGCT. a) Tubule with normal spermatogenesis contains cells expressing NANOS-1 (green) only. b) Tubule with a mixture of cells expressing AP-2γ (red; putative CIS cells) or NANOS-1 (putative 'normal' germ cells). c) Tubule with typical appearance of CIS with AP-2γ, (putative CIS cells) located on the basement membrane or NANOS-1 (putative 'normal' germ cells) located on the luminal side of the AP-2γ. Note that co-localised cells are not identified in CIS tubules. Sections counterstained with TO-PRO-3 (blue).

3.4.6. The expression of germ cell proteins in putative CIS cells that do not express classical CIS markers

In order to further characterise cells those germ cells within CIS tubules that express VASA, but do not express OCT4, triple fluorescent staining was performed for VASA, OCT4 and PLAP (Fig. 3.9). In tubules containing CIS cells the majority of cells expressed OCT4 and most of these were co-stained with PLAP. A small proportion of germ cells expressed VASA only. However there was also a population of germ cells that expressed VASA and PLAP, but did not express OCT4, suggesting that some of the $VASA^{+}/OCT^{-}$ cells may also represent CIS cells ($VASA^{+}/OCT^{-}/PLAP^{+}$). Cells with a $VASA^{+}/OCT^{-}/PLAP^{-}$ phenotype are likely to be 'normal' germ cells within these tubules. The expression profiles described for tubules from adults with TGCT are similar to those seen in normal human fetal germ cells (Fig. 3.10). During the second trimester the majority of the germ cells express OCT4. Some of these germ cells co-express OCT4 with PLAP. However there is not a population of cells that express PLAP with VASA, as described for a subpopulation of cells within CIS tubules (Fig. 3.9), consistent with the impression that these $VASA^{+}/OCT^{-}/PLAP^{+}$ cells are not 'normal' germ cells.

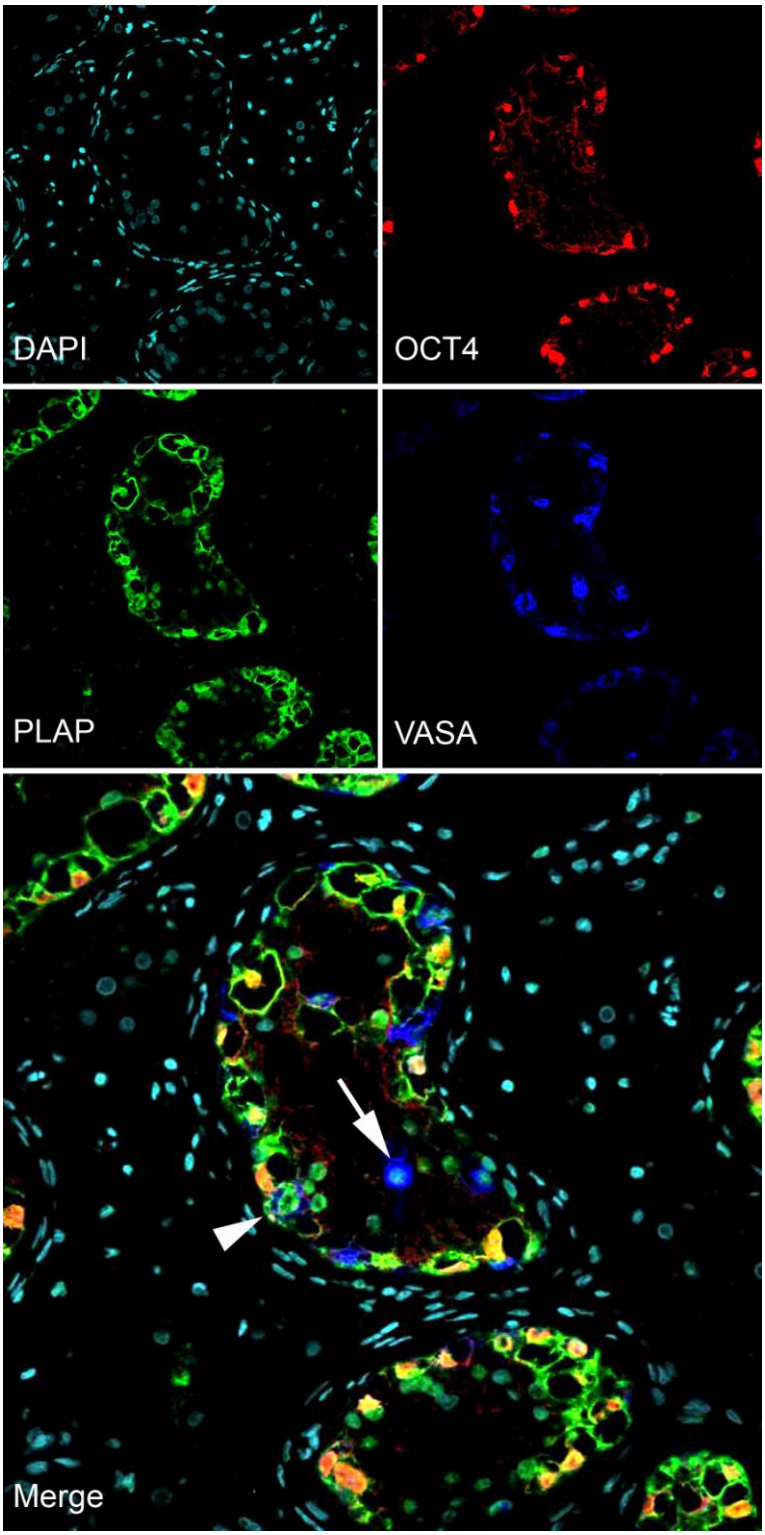

Figure 3.9. Expression of OCT4, PLAP and VASA in CIS containing tubules from a patient with a TGCT. Most of the cells co-express OCT4 (red) and PLAP (green). Some of the cells express VASA (blue; arrow) or VASA/PLAP (arrowhead), but remain negative for OCT4. Sections counterstained with DAPI (cyan).

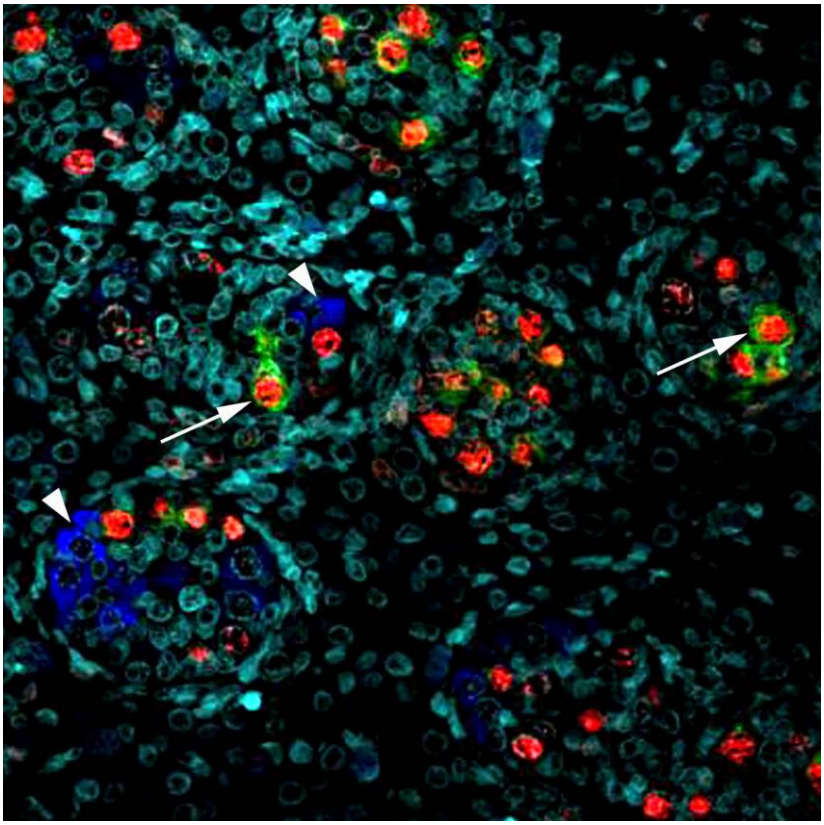

Figure 3.10. Expression of OCT4, PLAP and VASA in a 14 week human fetal testis. Most of the germ cells express OCT4 (red). Some of the cells express OCT4 and PLAP (green; arrow). None of the VASA positive cells (blue; arrowhead) expressed PLAP. Sections counterstained with DAPI (cyan).

3.4.7. Distinguishing between CIS cells and normal germ cells based on expression of a single marker

In order to distinguish between CIS cells and normal germ cells within CIS containing tubules, OCT4, PLAP and MAGE-A4 co-localisation was performed (Fig. 3.11). The majority of germ cells within CIS containing tubules expressed PLAP and OCT4. Germ cells that contained either or both of these markers were negative for MAGE-A4. MAGE-A4 was expressed in a small proportion of germ cells within these tubules and these could be regarded as normal germ cells retained within these CIS containing tubules.

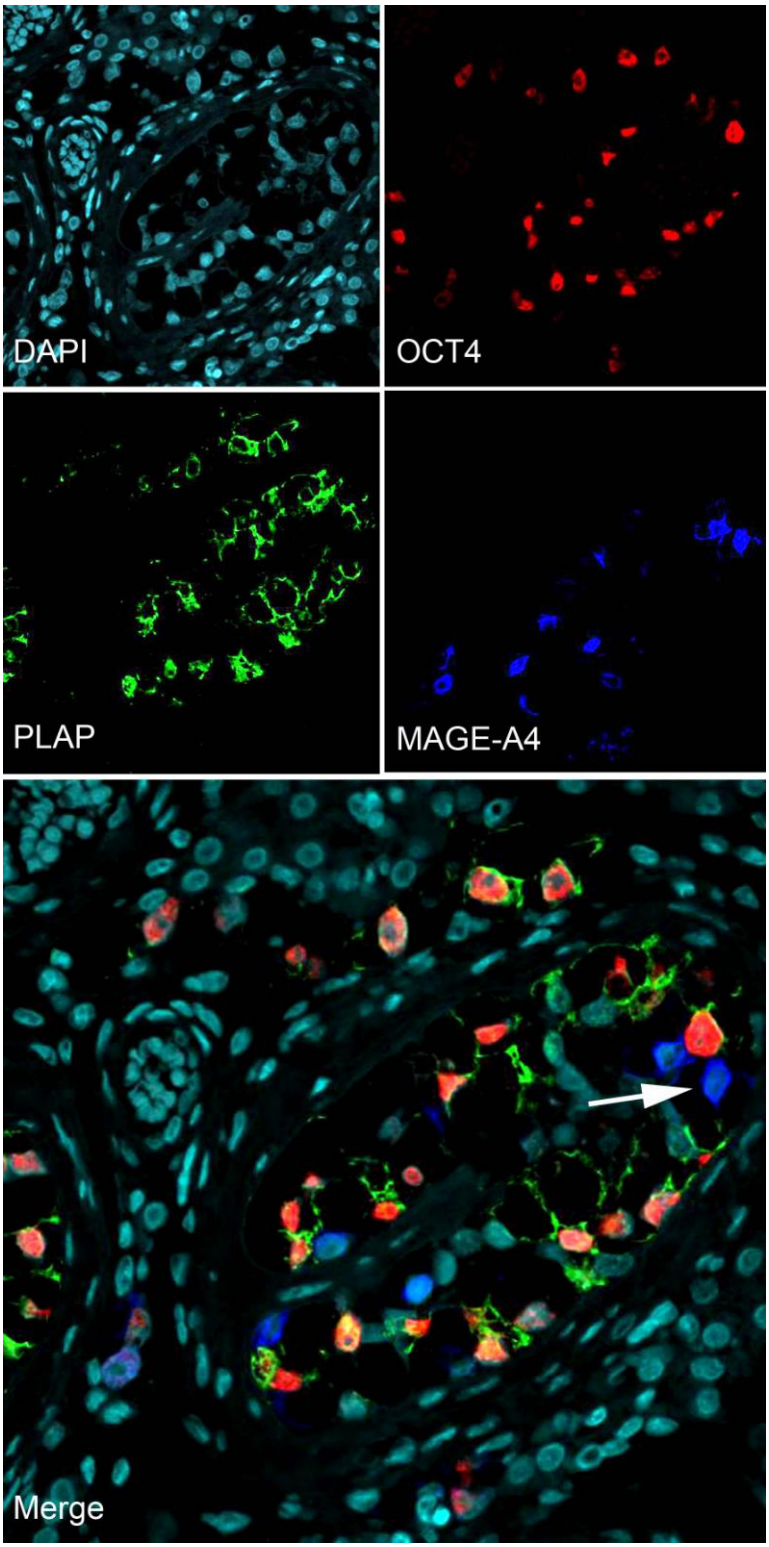

Figure 3.11. Expression of OCT4, PLAP and MAGE-A4 in tubules containing CIS from a patient with a TGCT. Most of the cells co-express OCT4 (red) and PLAP (green). Some of the cells express MAGE-A4 (blue; arrow) but remain negative for OCT4 and PLAP. Sections counterstained with DAPI (cyan).

Some rare germ cells expressed MAGE-A4 in combination with either PLAP or OCT4 (Fig. 3.12). Therefore although MAGE-A4 is mainly restricted to cells that do not express 'classical' CIS proteins it is not exclusive to these cells within CIS containing tubules.

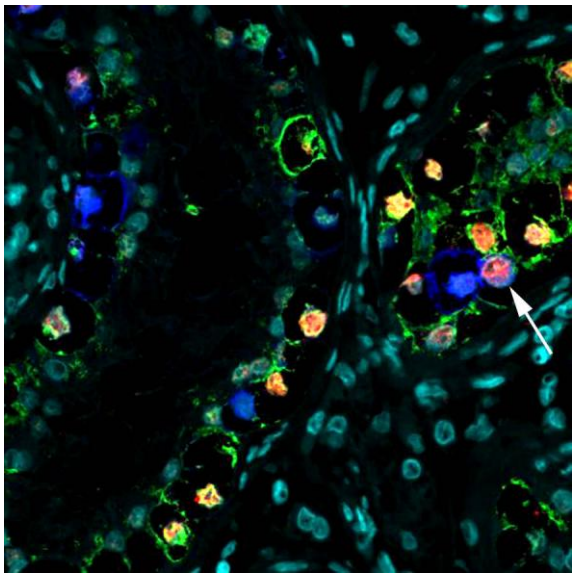

Figure 3.12. Co-expression of OCT4, PLAP and MAGE-A4 in CIS containing tubules from a patient with a TGCT. OCT4 (red) and MAGE-A4 (blue) are co-expressed (arrow) in some of the germ cells that do not express PLAP (green).

3.4.8. Subpopulations of CIS cells according to the expression of germ cell proteins and relationship with tumour type

The proportions of CIS cells with different phenotypes were quantified based on the expression of OCT4/PLAP/MAGE-A4 (Fig. 3.13). Only cells that expressed OCT4 or PLAP with typical morphological appearance of CIS cells were included to avoid counting normal germ cells within the CIS containing tubules. There was a significantly larger population of OCT4/PLAP CIS cells compared to CIS cells positive for either OCT4 or PLAP alone. These single stained cells were also significantly more frequent than any of the populations that expressed MAGE-A4.

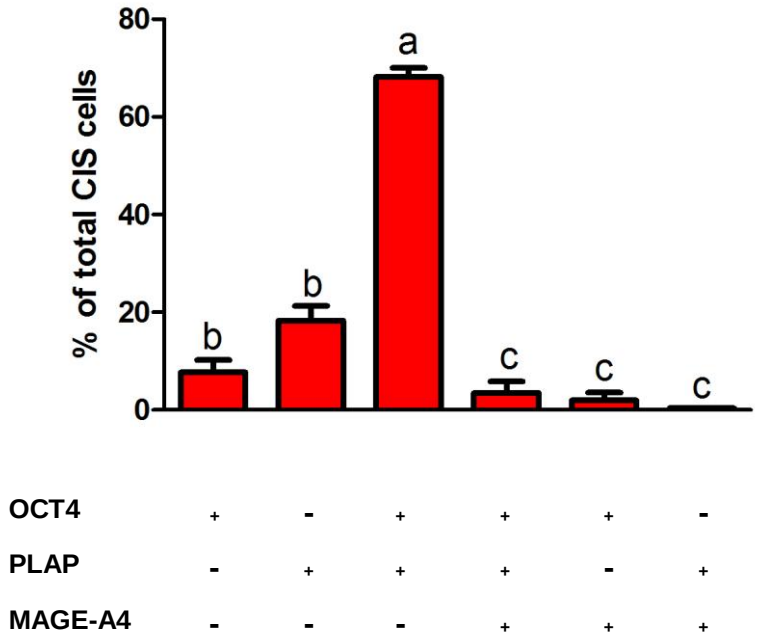

Figure 3.13. Quantification of subpopulations of CIS cells based on the expression of OCT4, PLAP and MAGE-A4. Groups without letters in common are significantly different from each other. Mean ± sem for n=9 (3 seminoma, 3 non-seminoma, 3 pre-invasive CIS), $p<0.05$.

We quantified the frequency of CIS subpopulations in the different tumour types (pre-invasive CIS, seminoma and non-seminoma). In all tumour types the most common phenotype was $OCT4^{+}/PLAP^{+}/MAGE\text{-}A4^{-}$. This profile was present in between 65 and 71% of CIS cells, whilst cells stained with either OCT4 or PLAP alone accounted for less than 25% of CIS cells in each group. CIS cells expressing MAGE-A4 were rare, accounting for less than 3% of cells in the pre-invasive and teratoma testis. Seminomas demonstrated a higher proportion of MAGE-A4 expressing cells than the other two groups with 14% of cells positive (Fig. 3.14). No statistically significant differences were observed for the expression of any of the subpopulations between the different tumour types.

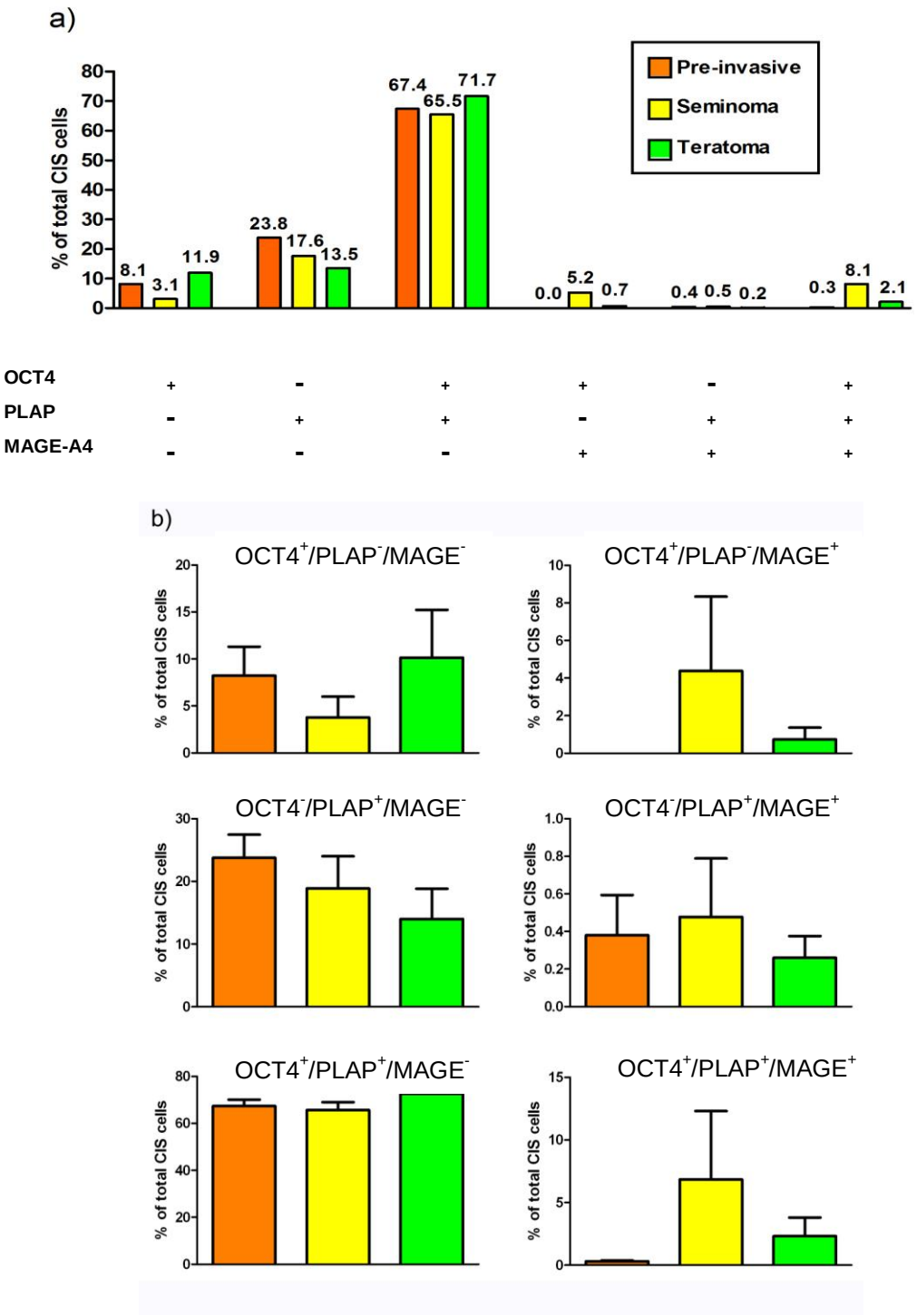

Figure 3.14. Quantification of CIS subpopulations based on expression of differentiation markers. a) Overview of quantification of subpopulations of CIS cells and comparison between tumour types. No significant differences are seen between tumour types for all subpopulations. Mean ± sem for n=3, $p>0.05$. b) quantification of subpopulations of CIS cells and comparison between tumour types. No significant differences are seen between tumour types for all subpopulations. Mean ± sem for n=3. $p>0.05$.

Overall, the proportion of CIS cells expressing MAGE-A4 was highest in the seminoma samples with a lower proportion in the teratoma samples (Fig. 3.15). Interestingly, the pre-invasive samples had almost no expression of MAGE-A4.

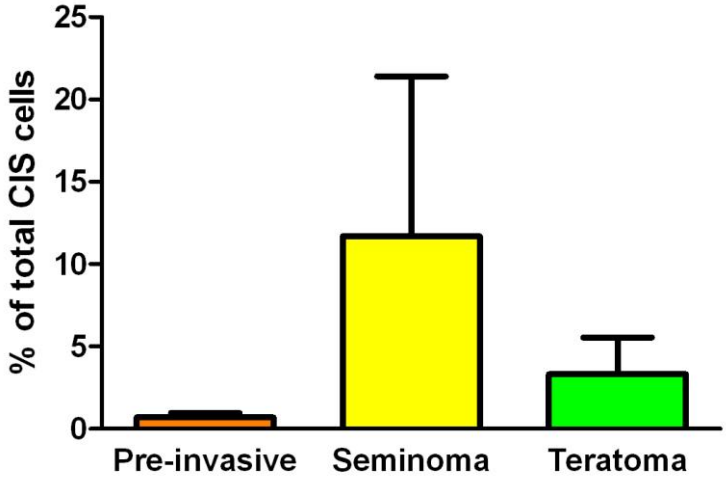

Figure 3.15. Quantification of MAGE-A4 expression in CIS cells and comparison between tumour types. No significant differences were seen between tumour types. Mean ± sem for n=3, $p>0.05$.

3.4.9. Comparison of differentiation status in CIS cells using two different methods

In order to compare the effectiveness of the different techniques employed in these studies, quantification of $OCT4^+/MAGE\text{-}A4^-$ and $OCT4^+/MAGE\text{-}A4^+$ CIS cells was performed on sections stained by either immunohistochemistry (IHC) or immunofluorescence (IF). The quantification of the two subpopulations was comparable using both techniques. MAGE-A4 expressing cells represented a mean of 4% of CIS cells for IHC compared to 12% of total CIS cells for IF (Fig. 3.16).

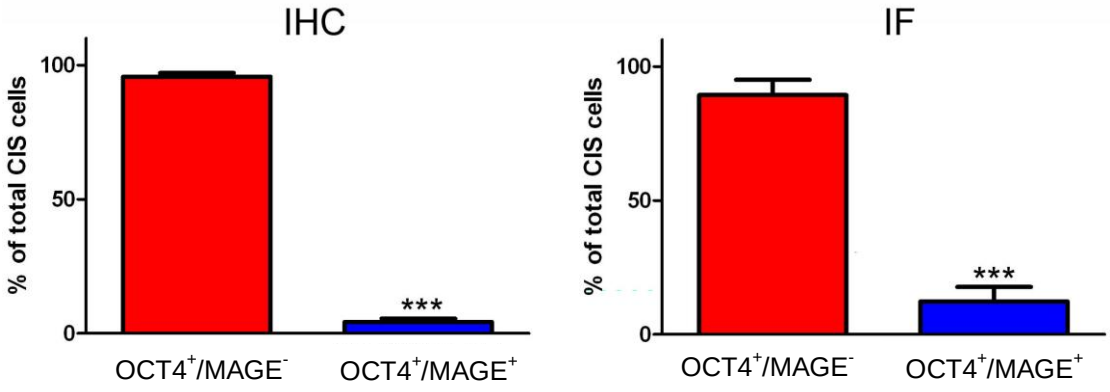

Figure 3.16. Comparison of immunohistochemistry and immunofluorescence for OCT4 v OCT4/MAGE-A4 expression in CIS cells. n=6 (3 seminoma, 3 teratoma) for each method. Mean ±sem. ***p<0.0001

3.4.10. Expression of proliferation markers in tubules containing CIS

We investigated the expression of proliferation markers Ki67, HH3 and PCNA in CIS tubules (Fig. 3.17). HH3 was expressed in a minority of germ cells within the CIS containing tubules, including cells that had the morphological appearance of CIS. A similar frequency of expression was also identified in tubules that had active spermatogenesis. PCNA was immunolocalised to the majority of the germ cells within CIS containing tubules and a large proportion of germ cells in tubules with active spermatogenesis, but was not detected in elongate spermatids. Ki67 staining was detected in more of the germ cells than HH3 but less than PCNA in both CIS tubules and tubules with active spermatogenesis.

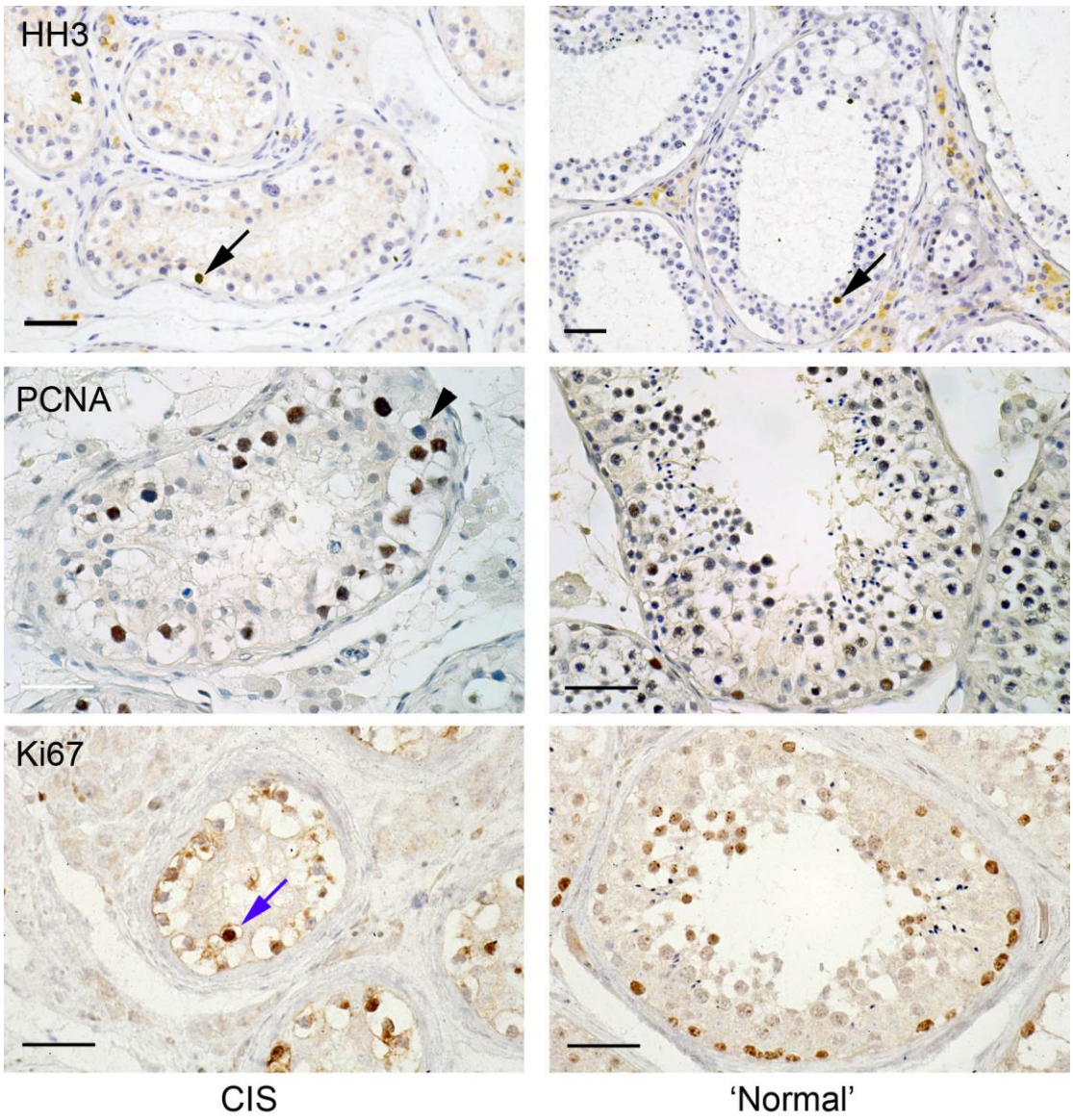

Figure 3.17. Expression of HH3, PCNA and Ki67 in CIS containing tubules and tubules exhibiting spermatogenesis with grossly 'normal' appearance. Occasional cells are positive for HH3 (top panels; black arrows). PCNA (middle panels) negative CIS cells were rare (arrowhead). A Ki67 (lower panels) positive germ cell without typical CIS appearance, which may represent a differentiating germ cell can also be identified (blue arrow). Original magnification x20 (upper panels) and x40 (middle and lower panels). Scale bar - 50μm.

3.4.11. Proliferation within subpopulations of CIS cells

To obtain quantitative data for proliferation in the various subpopulations of CIS cells, Ki67 was chosen as the proliferation marker because it is expressed in all cells

that are not resting and therefore represented a suitable marker of germ cell proliferation (1.15). HH3 only stained a very small number of cells, whilst Ki67 stained enough cells to allow quantification studies. PCNA stained almost all the cells, which made it unsuitable for quantification studies. Co-staining of MAGE-A4 and Ki67 in CIS tubules revealed that both MAGE-A4 positive and MAGE-A4 negative cells may be proliferating. In addition there were also cells that were not proliferating in the MAGE-A4 positive and negative populations (Fig. 3.18).

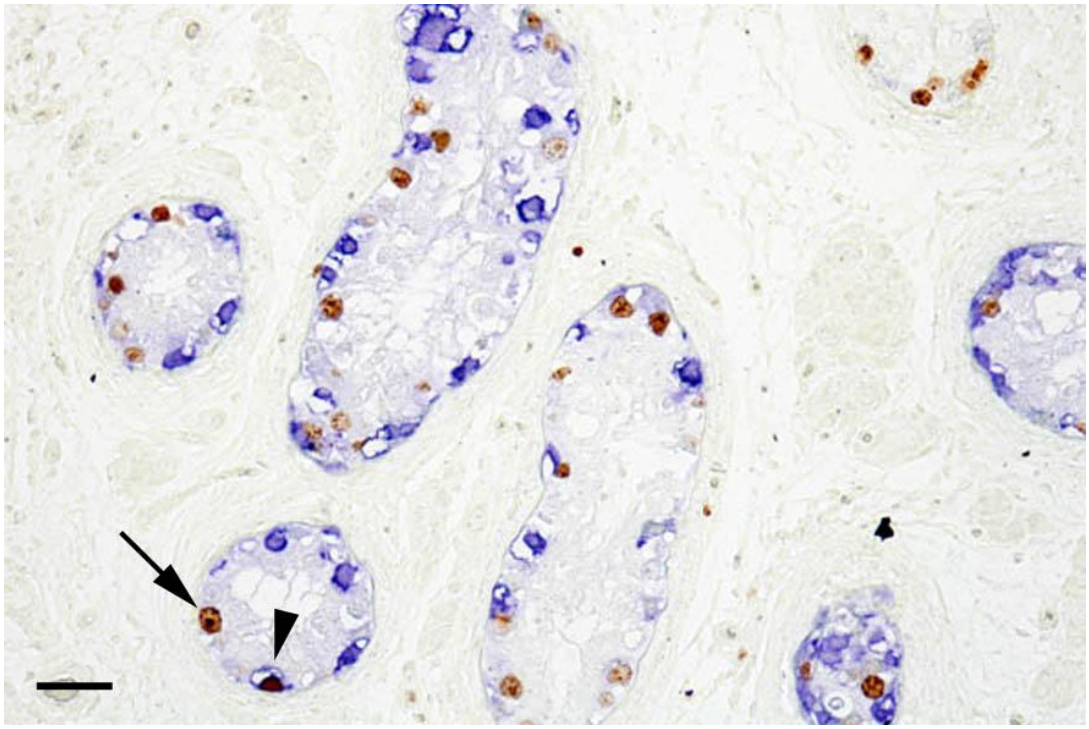

Figure 3.18. Co-expression of Ki67 and MAGE-A4 in CIS containing tubules. Proliferating (KI67, brown) cells may be MAGE-A4 (blue) positive (arrowhead) or MAGE-A4 negative (arrow). Note that there are also non-proliferating cells that may be either positive or negative for MAGE-A4 and that all four types of cell can be identified in the same tubule. Scale bar - 50μm.

Proliferative activity of CIS cells may reflect their capacity to develop into an invasive tumour. Therefore the proliferative activity of the various subpopulations of CIS cells was evaluated. Using triple staining for OCT4/MAGE/Ki67, CIS cells could be identified by the expression of OCT4 (Fig. 3.19). Within the OCT4

expressing cell population there were MAGE-A4 positive and MAGE-A4 negative cells. A proportion of both $OCT4^+/MAGE\text{-}A4^-$ and $OCT4^+/MAGE\text{-}A4^+$ cells were Ki67 positive (Fig. 3.19).

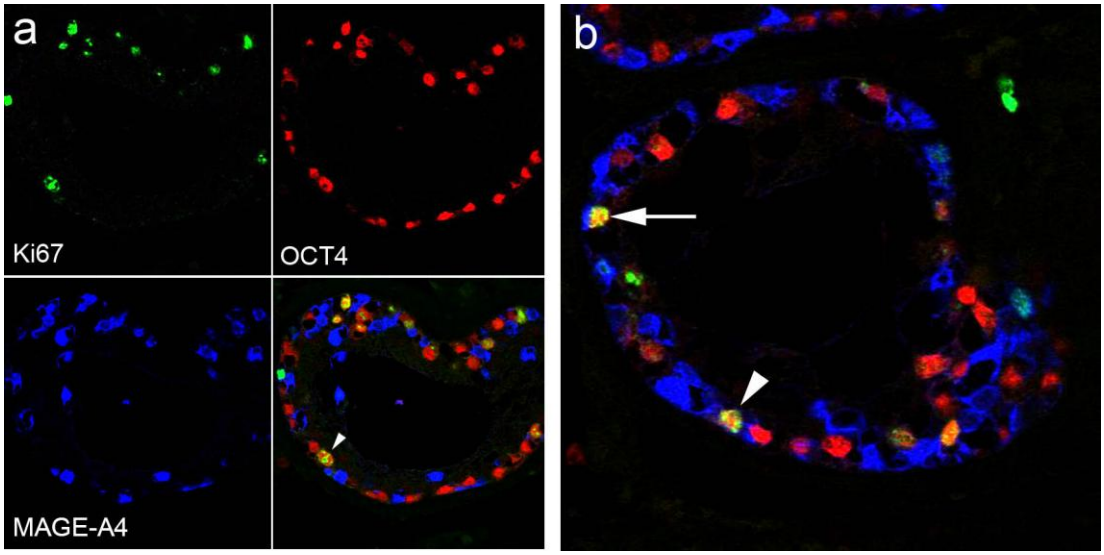

Figure 3.19. Co-expression of Ki67, MAGE-A4 and OCT4 in CIS containing tubules. OCT4 (red) positive cells can be considered CIS cells, whilst MAGE-A4 (blue) may represent CIS or differentiating germ cells. a) proliferating (Ki67, green) $OCT4^+/MAGE\text{-}A4^-$ cells can be seen (arrowhead). b) Proliferating $OCT4^+/MAGE\text{-}A4^-$ (arrowhead) and $OCT4^+/MAGE\text{-}A4^+$ (arrow) cells can be seen in the same tubule.

3.4.12. Variation in proliferation of CIS cells depending on tumour type

The proportion of CIS cells that were proliferating was calculated for the different tumour types, based on the co-expression of OCT4 and Ki67. The proliferation index was highest in CIS cells from patients with pre-invasive disease (15%). CIS cells within testes from patients with seminoma had a lower proliferation index (12%), whilst those patients with teratomas had the lowest proportion of proliferating CIS cells (9%). There was no significant difference in proliferation rate between the different tumour types (Fig. 3.20).

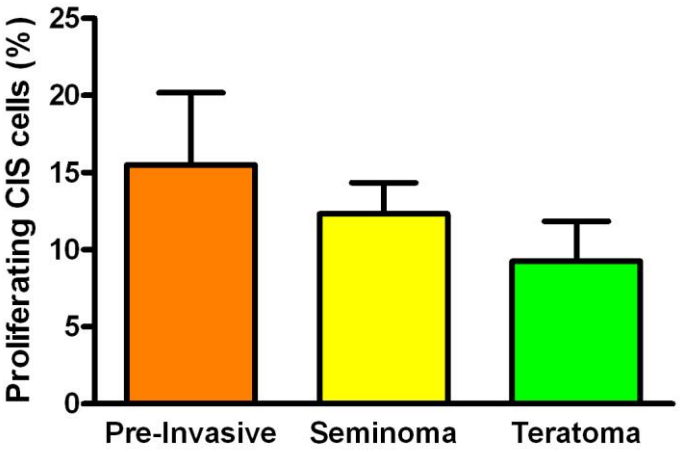

Figure 3.20. Proliferation index for CIS cells in pre-invasive CIS, seminoma and teratoma. n=3. Mean ± sem for n=3, p>0.05.

The proliferation index in the different subpopulations of CIS was also calculated. MAGE-A4 expressing CIS cells ($OCT4^+/MAGE\text{-}A4^+$) had a significantly lower proliferation index than the CIS cells that did not express MAGE-A4 ($OCT4^+/MAGE\text{-}A4^-$) (Fig. 3.21).

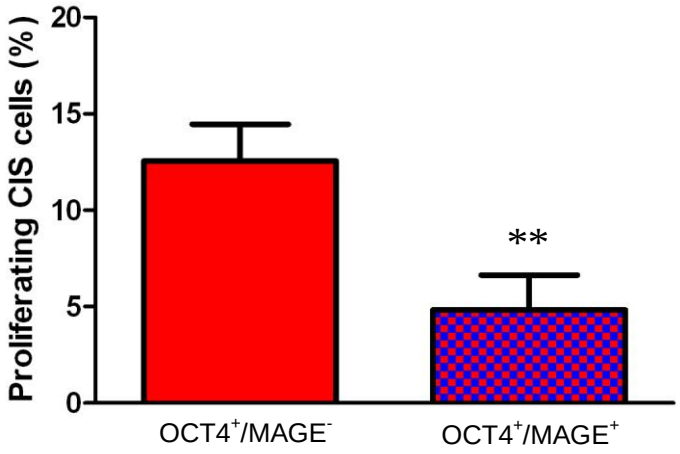

Figure 3.21. Proliferation index for MAGE-A4 expressing CIS cells. Quantification of MAGE-A4 expressing CIS cells (OCT4+MAGE-A4) and comparison with CIS cells that do not express MAGE-A4 (OCT4). Mean ± sem for n=9. **p<0.01.

When the individual tumour types were analysed the same trend could be seen for pre-neoplastic, seminoma and teratoma samples, with a significant difference in the

proliferation index of the CIS subpopulations only in samples from patients with seminoma (Fig. 3.22).

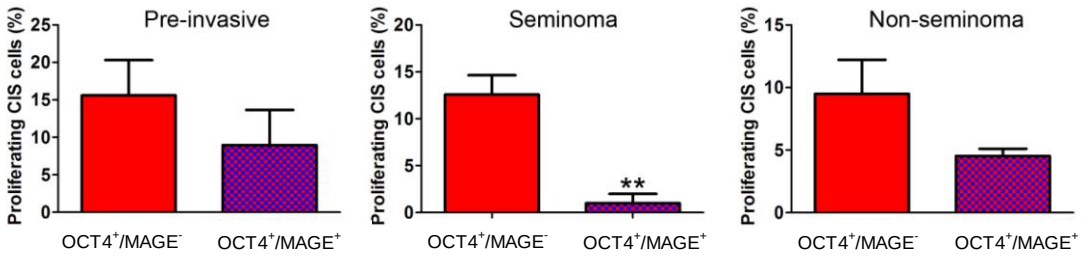

Figure 3.22. Proliferation index for MAGE-A4 expressing CIS cells in individual tumour types. Quantification of proliferation MAGE-A4 expressing CIS cells ($OCT4^+/MAGE\text{-}A4^+$) and comparison with those not expressing MAGE-A4 ($OCT4^+/MAGE\text{-}A4^-$), in individual tumour types. Mean ± sem for n=3. **p<0.01.

3.4.13. Sertoli cells within CIS tubules express markers of undifferentiated cells

Sertoli cells in the normal fetal testis are immunopositive when stained with a pan-cytokeratin antibody. The expression of protein(s) detected by this antibody is lower in postnatal testes and Sertoli cells in the adult testis are immunonegative. Therefore immunostaning can be used as a marker of undifferentiated Sertoli cells (Fig. 3.23). Androgen receptor is immunolocalised to the peritubular myoid and some interstitial cells in the fetal testis and this continues into adulthood. Androgen receptor was not expressed in the Sertoli cells of the human fetal testis, but it was detected in adult Sertoli cells.

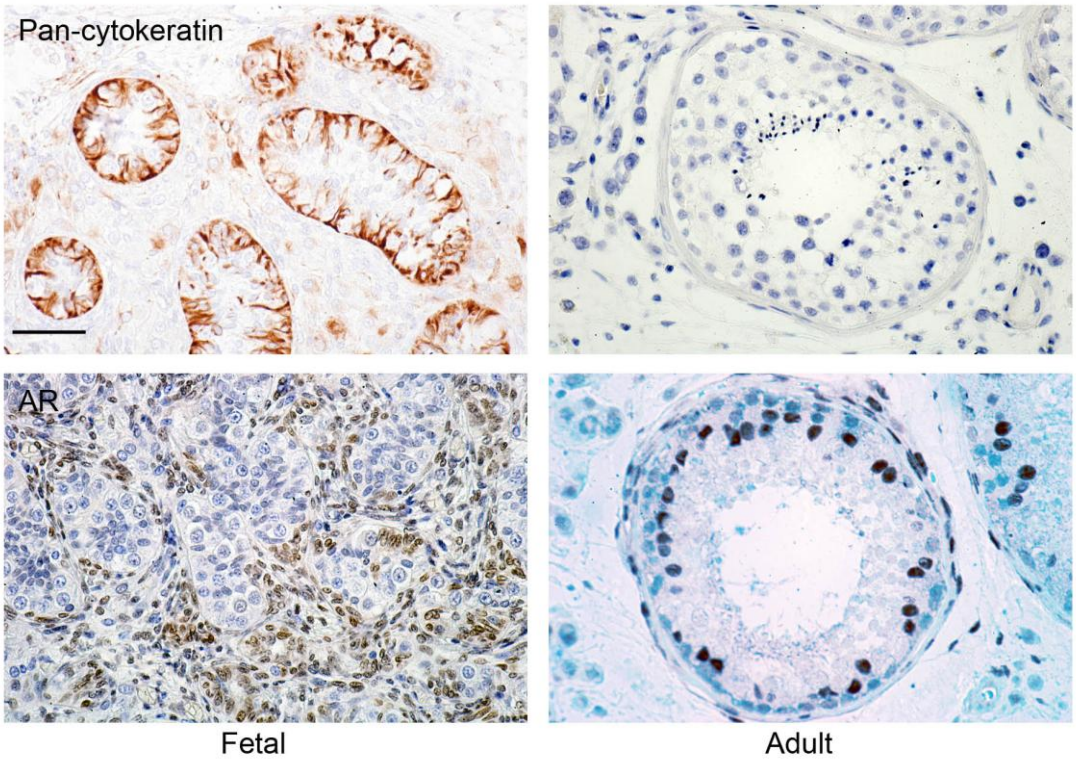

Figure 3.23. Expression of pan-cytokeratin and androgen receptor in fetal and adult human testis. Pan-cytokeratin (top panels) is expressed in fetal Sertoli cells, but not those of the adult. Androgen receptor (lower panels) is expressed in Sertoli cells of the adult, but fetal Sertoli cells are negative for androgen receptor. Scale bar - 50μm.

The differentiation status of Sertoli cells was investigated within CIS containing tubules, using co-staining for androgen receptor and pan-cytokeratin (Fig. 3.24). Androgen receptor was immunolocalised to all the Sertoli cells of CIS tubules, however pan-cytokeratin was only detected in a subpopulation of these Sertoli cells. The tubules expressing cytokeratin tended to be in regions adjacent to the tumour itself, whilst cytokeratin was often not detected in regions distant from the tumour. A similar expression pattern was seen for both seminomas and teratomas.

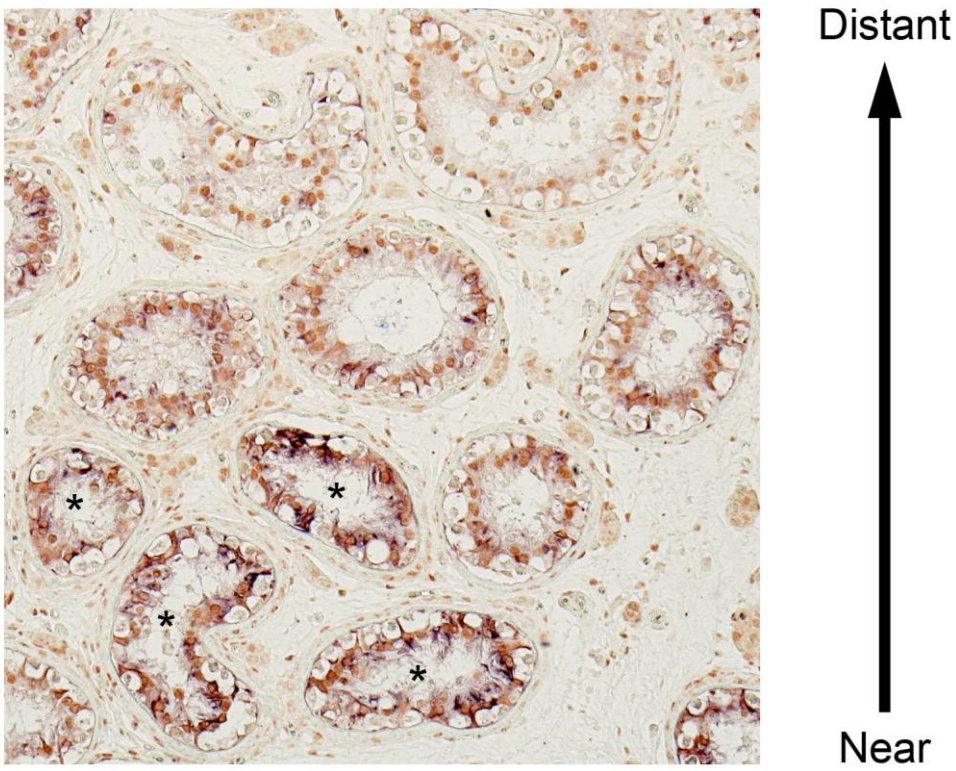

Figure 3.24. Co-expression using pan-cytokeratin and androgen receptor antibodies on sections of seminiferous tubules from a patient with a TGCT. Strong cytokeratin (blue) expression is shown in Sertoli cells in tubules (*) adjacent to the tumour (near), but not in those further away the tumour (distant), whilst androgen receptor (brown) is expressed in all Sertoli cells. Scale bar - 50μm.

3.4.14. The relationship between CIS cells and their associated Sertoli cells

In order to investigate the relationship between the CIS cells and the differentiation status of their associated Sertoli cells sections were co-stained for OCT4 and cytokeratin (Fig. 3.25). OCT4 expression was seen in most of the cells within tubules of both seminoma and teratoma patients, regardless of the distance from the tumour. Immunoexpression of Pan-cytokeratin was more frequent in the Sertoli cells of CIS tubules adjacent to the tumour. However there was no apparent association between the differentiation status of the CIS cells and the Sertoli cells that surrounded them, based on detection with the pan-cytokeratin antibody (Fig. 3.25).

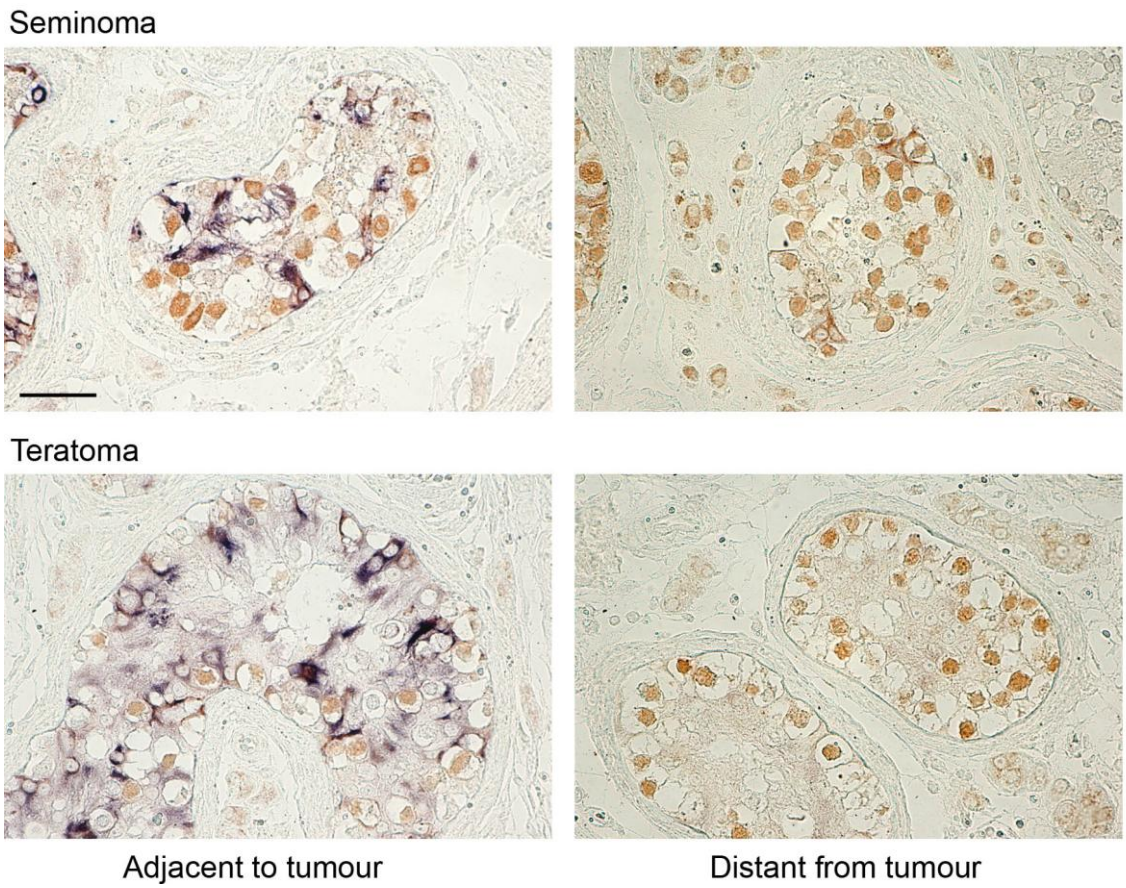

Figure 3.25. Co-expression of pan-cytokeratin and OCT4 in CIS tubules from patients with TGCT. OCT4 (brown) is expressed in CIS cells regardless of the pan-cytokeratin (blue) status of its associated Sertoli cell. Scale bar - 50μm.

Immunolocalisation of VASA and pan-cytokeratin also demonstrated that the differentiation status of the CIS cells was not associated with the differentiation status of the Sertoli cells within a CIS tubule. VASA positive cells were located in CIS containing tubules regardless of whether those tubules expressed pan-cytokeratin (Fig. 3.26)

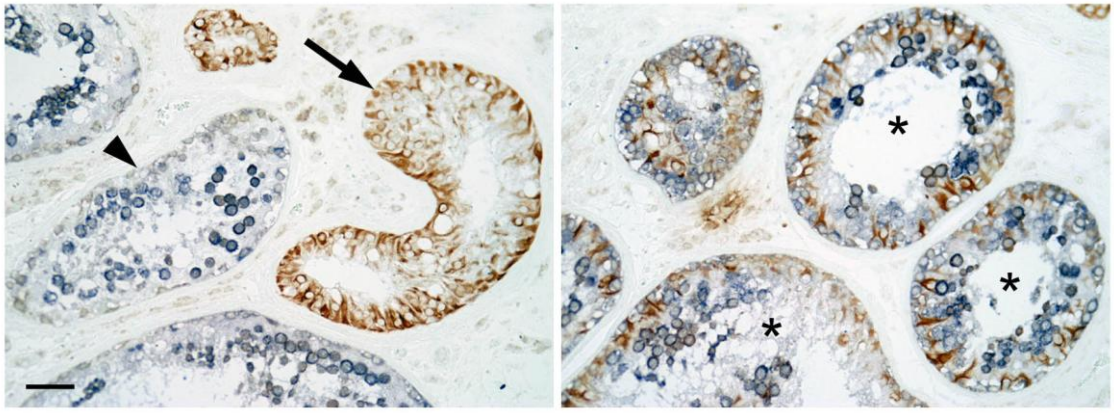

Figure 3.26. Co-expression of pan-cytokeratin and VASA in CIS tubules from a patient with a TGCT. Tubules containing VASA positive germ cells (blue) are present (arrowhead), as are tubules with pan-cytokeratin only (brown; arrow). Tubules expressing both markers (*) were also identified. Scale bar - 50μm.

3.5. Discussion

3.5.1. Heterogeneous expression of germ cell proteins in CIS

3.5.1.1. CIS cells do not always express classical markers

OCT4 and PLAP are described as 'classical' markers of CIS cells and are routinely used for diagnostic purposes for patients at risk of, or with suspected, TGCT (Oosterhuis and Looijenga, 2005). PLAP has been reported to be detectable in 83-99% of CIS specimens, whilst OCT4 is reported to be a sensitive and specific marker (Jones et al., 2004), and present in all CIS cells (Oosterhuis and Looijenga, 2005);(van Casteren et al., 2008). In addition the expression of NANOG has been reported as present in an identical population of germ cells to that of OCT4 (Oosterhuis and Looijenga, 2005). The present co-localisation studies demonstrate that most of the cells expressing either NANOG or PLAP, also express OCT4. Moreover OCT4 and PLAP were co-localised in 68% of CIS cells. However, surprisingly there was also a population of cells with a CIS phenotype that expressed either PLAP or NANOG, but were immunonegative OCT4 expression. In the case of the $PLAP^{+}/OCT4^{-}$ population this was quantified as representing 18% of the total CIS cells. NANOG positive cells that did not express OCT4 were much less frequent. Occasional

gonocytes have also been described in the normal human fetal testis that are $NANOG^{+}/OCT4^{-}$ (Kerr et al., 2008). In the literature there has been some debate about the choice of OCT4 antibody and the specificity of antibodies from different sources (Looijenga, 2009). OCT4 expression has been reported in spermatogonia of the mouse (Pesce et al., 1998). OCT4 has also been reported to be present in the adult stem cell spermatogonia of the human, however these reports may be explained by the use of non-specific antibodies (Looijenga, 2009). For example the OCT4 antibodies used in the present studies were polyclonal goat antibodies directed against either the N-terminus or C-terminus of the OCT4 protein and produced the same results, with no staining found in normal adult tissues or negative controls. The present results demonstrate heterogeneity in expression of markers that are considered highly sensitive and specific for CIS cells. This highlights the fact that a single marker cannot be used to definitively determine whether an individual cell is a CIS cell. This is particularly important for interpretation of studies that rely on immunostaining for OCT4 as an indication that a cell is not a CIS cell. In a study of non-seminomas a population of cells that are $VASA^{+}/OCT4^{-}$ was described. The authors suggested that this population could have arisen as a result of differentiation of non-seminoma into germ cells (Honecker et al., 2006). However results obtained during the present study would imply that these cells could actually be OCT4 negative CIS cells.

3.5.1.2. CIS cells do not always display a differentiation phenotype characteristic of fetal germ cells

CIS cells have been postulated to arise from embryonic germ cells and this is based on evidence from a combination of morphological, immunohistochemical and genetic studies (Rajpert-De Meyts, 2006);(Looijenga et al., 2007a);(Almstrup et al., 2004). Expression of proteins implicated in maintenance of pluripotency such as NANOG and OCT4, can be demonstrated in human fetal gonocytes (Gaskell et al., 2004);(Rajpert-De Meyts et al., 2004);(Hart et al., 2005). In addition proteins associated with migrating and early fetal germ cells such as AP2γ (Hoei-Hansen et

al., 2004) and PLAP (Jorgensen et al., 1995);(Gaskell et al., 2004) have also been demonstrated in the same cell population. The proportions of germ cells expressing these proteins is reduced as gestation progresses and the germ cells differentiate (Hoei-Hansen et al., 2005);(Mitchell et al., 2008);(Rajpert-De Meyts, 2006) (Cools et al., 2005);(Rajpert-De Meyts et al., 2004).

The same proteins have been described as being frequent markers of CIS cells (Hoei-Hansen et al., 2004);(Looijenga et al., 2007a);(Jorgensen et al., 1995);(Hart et al., 2005). However, co-localisation of combinations of these proteins have revealed populations of CIS cells that do not express a phenotype that correlates to a particular stage of normal fetal germ cell development. For example, PLAP can be detected in most of the germ cells in a first trimester fetal testis, but is rare by the start of the second trimester (Honecker et al., 2004). OCT4 is also present in most of the germ cells of the first trimester, but is downregulated later in gestation in comparison to PLAP (Gaskell et al., 2004). The present study has identified $OCT4^{-}$/$PLAP^{+}$ cells with a phenotype characteristic of CIS, which contrasts with reports of expression in human fetal testis where PLAP is always co-expressed with OCT4 (Honecker et al., 2004).

The fact that the majority of CIS cells are immunopositive for proteins that are found to be expressed in germ cells in normal human fetal gonads, would support the hypothesis that CIS cells originate from human fetal germ cells, however the finding of phenotypes that do not occur in the normal human fetal testis suggests that some changes in expression occur after transformation has taken place.

3.5.1.3. Distinguishing between normal germ cells and CIS cells within the same tubule

In addition to the proteins that can be detected in PGCs and gonocytes, CIS cells have also been reported to express proteins characteristic of more differentiated germ cell types such as VASA (Rajpert-De Meyts et al., 2003) and MAGE-A4 (Aubry

et al., 2001). These proteins are first expressed in germ cells during fetal life and can be detected in an increasing proportion of germ cells within the individual testis cords as the cells differentiate (Aubry et al., 2001);(Castrillon et al., 2000);(Gaskell et al., 2004). Both proteins are readily detected in postnatal germ cells where MAGE-A4 expression is present in pre-meiotic cells (Aubry et al., 2001) and VASA is detected in both pre and post-meiotic germ cells (Castrillon et al., 2000).

MAGE-A4 (Aubry et al., 2001) and VASA are have also been reported as present in a proportion of CIS cells (Rajpert-De Meyts et al., 2003). Because CIS tubules, also contain Sertoli cells and may contain germ cells that are not strictly CIS cells, it is not possible to say with certainty whether the MAGE-A4 and VASA population represent the CIS, non-CIS or both populations. The present study has provided novel information of the phenotype of CIS cells based on the use of double and triple immunohistochemistry and fluorescence. Co-expression of 'classical' CIS markers such as OCT4, PLAP and AP2γ with markers of differentiated germ cells (VASA and MAGE-A4) revealed that there are a proportion of cells that express both markers. These are likely to be CIS cells because OCT4, PLAP and AP2γ are not normally expressed in the adult human testis (Looijenga, 2009);(Hoei-Hansen et al., 2004);(Giwercman et al., 1991);(Skakkebaek et al., 1987). However MAGE-A4 and VASA are only expressed in a small proportion of CIS cells. We have quantified the expression of these differentiated germ cell markers in CIS for the first time. MAGE-A4 is only expressed in 6% of CIS cells and is therefore not a common phenotype for CIS cells. Heterogeneous expression of MAGE-A4 in CIS has been described previously, however VASA was reported to be expressed in all CIS cells (Rajpert-De Meyts, 2006). Our findings appear at odds with this conclusion as VASA expression was not found in all CIS cells in the present study and was expressed heterogeneously in a similar fashion to MAGE-A4.

In a previous study, co-staining for OCT4 and VASA identified cells that had an $OCT4^{-}/VASA^{+}$ phenotype (Cools et al., 2005). Based on this pattern of staining these

cells might have been identified as differentiated germ cells rather than CIS cells, however the use of triple co-localisation has revealed that some cells that do not express OCT4 may express PLAP and therefore these $OCT4^{-}/VASA^{+}$ cells can still be considered as CIS cells. The present results suggest that only those germ cells that are $OCT4^{-}/PLAP^{-}$ represent a 'differentiated' germ cell population that have not undergone malignant transformation.

3.5.1.4. The frequency of CIS subpopulations varies between pre-invasive disease, seminoma and teratoma

The use of tissue from patients who have already developed a TGCT make it impossible to determine whether CIS cells arise from different subpopulations of fetal germ cells, or alternatively whether there is a common fetal cell of origin and subsequent changes in expression occur with CIS cell progression. One approach to addressing some of these questions is to use tissue from patients with pre-invasive disease. CIS cells are believed to lie dormant within the testis for years during childhood before they develop into an invasive tumour (Skakkebaek, 1972b). From puberty onwards these cells may transform into either a seminoma or a non-seminoma. It is unclear as to whether there is a specific difference between the CIS cell phenotype in seminoma or non-seminoma. Because seminoma cells have also been described as the malignant equivalent of the fetal germ cell, it may be hypothesised that MAGE-A4 expression would be higher in the CIS cells that are associated with these tumours than in the non-seminomas, in which the malignant cells are differentiated somatic and extra-embryonic tissues. A trend towards increased MAGE-A4 expression was found in CIS cells from the seminoma patients, however the expression was variable between individual patients and the difference was not statistically significant. The fact that the CIS cells within the non-seminomas all expressed very low levels of MAGE-A4 may point to a requirement of MAGE-A4 for the formation of a seminoma and this would be supported by the fact that seminoma cells variably express MAGE-A4, whilst non-seminomas and the preceding embryonal carcinoma stage do not (Rajpert-De Meyts, 2006) (Aubry et al.,

2001). MAGE-A4 is not expressed in the gonocyte and therefore if seminoma is the malignant counterpart of the gonocyte, then MAGE-A4 positive cells within these tumours could represent subsequent differentiation of CIS cells, de-differentiation of MAGE-A4 expressing spermatogonia, or alternatively the presence of 'differentiated' MAGE-A4 positive germ cells within the tumour. Pre-invasive CIS cells also rarely expressed MAGE-A4 in a similar proportion of cells to the non-seminomas. This again could suggest that the expression of MAGE-A4 could direct the CIS cells towards a seminoma. The concept of heterogeneous cell populations having a different developmental potential has been suggested for SSC in the mouse testis, where a fraction of these cells express OCT4 (Buageaw et al., 2005). However, the fact that we have only investigated small numbers of samples and that we cannot predict which of the tumour types will result from the pre-invasive samples analysed, make it difficult to reach firm conclusions.

3.5.2. Proliferation rate varies between the different CIS subpopulations

Proliferation is one of the characteristic features of malignant cells. The switch from pre-invasive CIS to invasive tumour is thought to result from an increase in seminiferous tubule diameter by proliferation of CIS cells (Donner et al., 2004). Proliferation in the various stages of TGCT development has been investigated by quantifying mitotic frequency. CIS cells are reported to have a mitotic frequency of 0.65% which increases through intratubular seminoma (0.84%) to seminoma with a mitotic frequency of 3.59% (Hofken and Lauke, 1996), suggesting that the cells with the highest malignant potential are more proliferative. Interestingly when proliferation was investigated in the different tumour types in the present studies, the highest rate was found in the CIS cells from the pre-invasive samples. This was not entirely expected as the CIS cells are described as being relatively inactive during this period. It was also demonstrated that $OCT4^+/MAGE\text{-}A4^-$ CIS cells had a significantly higher expression of Ki67 than the $OCT4^+/MAGE\text{-}A4^+$ CIS cells, suggesting that the $MAGE^-$ population of CIS cells are more proliferative. The persistent expression of genes such as OCT4, TSPY and CCND2 have previously

been implicated in the survival and expansive proliferation of CIS cells (Cools et al., 2006b);(Rajpert-De Meyts, 2006). This result is also in keeping with a previous report that describes increased malignant potential and aggressiveness in OCT4 expressing cells in tumours derived from ES cells (Gidekel et al., 2003). In these tumours, co-localisation of OCT4 with Ki67 showed high proliferative activity in the OCT4 expressing cells, with no proliferation demonstrated in the OCT4 negative areas (Gidekel et al., 2003). Therefore reduced proliferation may be a feature of cells that are further down the differentiation pathway from $OCT4^{+}$/MAGE-$A4^{-}$ cells. This concept is supported by the fact that in the normal human fetal testis a significantly increased proportion of $OCT4^{+}$/MAGE-$A4^{-}$ cells express Ki67, compared to the MAGE-$A4^{+}$ population. These results are consistent with a previous study in normal human fetal testis that described Ki67 expression in most KIT positive cells, but only some of the MAGE-A4 expressing cells (Pauls et al., 2006). These results will be discussed in more detail in chapter 5 (Fig. 5.23).

3.5.3. Sertoli cell differentiation status varies within CIS tubules

3.5.3.1. Differentiation status of Sertoli cells is associated with tumour proximity

Sertoli cells located within CIS tubules displayed variable expression of AR and cytokeratin proteins used as markers of Sertoli cell maturity. Immunopositive staining for cytokeratin was found in some tubules; Sertoli cells expressing this marker were often located close to the tumour itself, whilst Sertoli cells in tubules further away were frequently immunonegative for this marker. The pan-cytokeratin antibody used in this study recognises cytokeratins 1,4,5,6,8,10,13,18, and 19. A similar gradient has been described for cytokeratins 8, 18 and 19, with expression being more abundant in atrophic tubules adjacent to tumours (Nistal et al., 2006). The current study has also discovered tubules with Sertoli cells that are immunopositive for cytokeratin and androgen receptor. This would support the concept of either a partial differentiation or a partial de-differentiation of Sertoli

cells, because cytokeratin 18 expression ceases in late gestation, whilst androgen receptor is not expressed until late childhood (Brehm et al., 2006). Partial differentiation may have occurred such that androgen receptor is expressed as normal, but cytokeratin has been retained from fetal life. Alternatively partial de-differentiation may have occurred in Sertoli cells that had previously undergone differentiation and ceased to express cytokeratin, only to re-express cytokeratin later (Fig. 3.27). Evidence exists to support the concept of de-differentiation of Sertoli cells. In rhesus monkeys, cytokeratin 18 expression can be induced in the testis by making the adult animal cryptorchid (Zhang et al., 2004) and a similar result is obtained by local heating of the testis (Zhang et al., 2006). The re-expression of cytokeratin 18 is reversible and expression is gradually reduced with time and eventually ceases. Sertoli cells in prepubertal dysgenetic testes, express AMH but barely any CK18 (Schreiber et al., 2003). These patients are at high risk of CIS and TGCT and therefore the appearance of cytokeratin in CIS containing tubules in these patients would indicate that these cells have undergone initial differentiation and subsequent de-differentiation to re-express cytokeratin. The finding that cytokeratin can still occasionally be found in CIS tubules distant to the tumour itself supports the hypothesis that it is a primary event within the Sertoli cells, rather than a reactive event (Nistal et al., 2006). It is unclear whether the CIS cells themselves are the trigger for this primary failure.

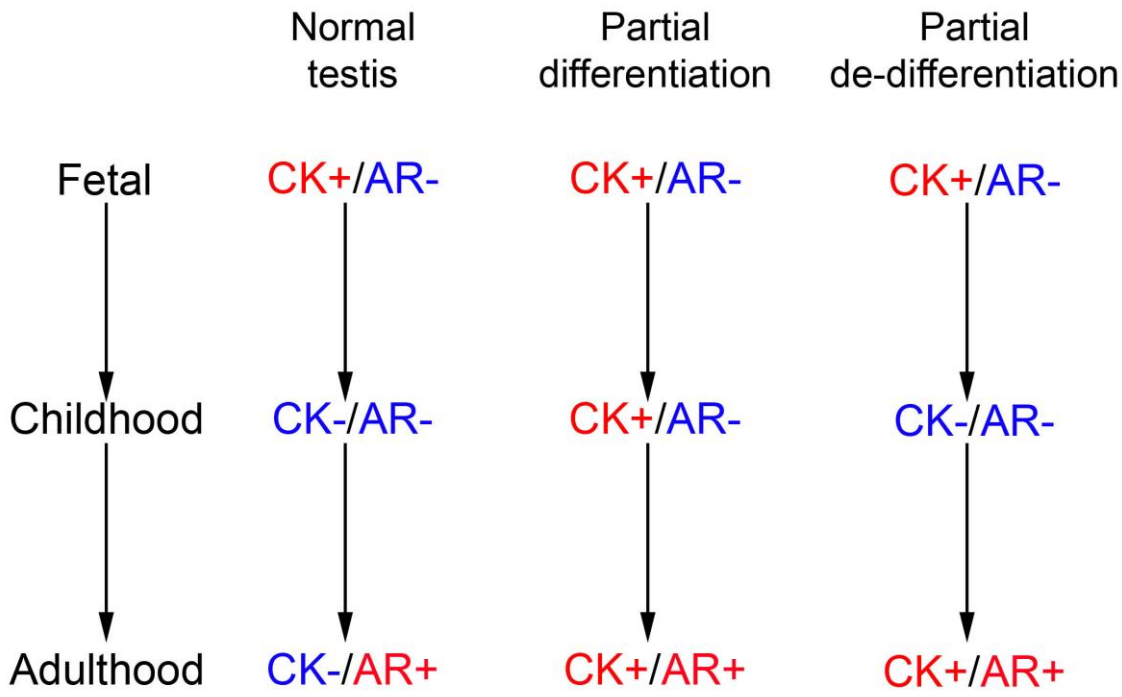

Figure 3.27. Expression of cytokeratin and androgen receptor in the normal testis and in models of partial differentiation or de-differentiation. In partial differentiation androgen receptor (AR) is expressed in a normal fashion but cytokeratin (CK) expression is retained. In the partial de-differentiation model loss of CK and expression of AR occurs as in the normal testis, but CK is subsequently re-expressed while AR is retained.

3.5.3.2. Differentiation status of Sertoli cells is not associated with that of the neighbouring CIS cell

Sertoli cells play a crucial role in supporting germ cell maturation during spermatogenesis. The relationship between the differentiation status of the Sertoli cell and that of the associated CIS cell is unclear. This study hypothesised that the Sertoli cells expressing markers of de-differentiation are associated with tubules that contain CIS cells only, whilst those Sertoli cells that are negative for these markers are associated with tubules that contain a mixture of CIS and differentiating germ cells. Previous studies have suggested that the presence of differentiating germ cells within the CIS tubules prevents the expression of cytokeratin within CIS containing tubules (Kliesch et al., 1998) and that cytokeratin 18 expression is only present in CIS tubules that do not contain normal germ cells (Brehm and Steger, 2005).

However in the present studies no relationship was found between the Sertoli cell differentiation status and the CIS/germ cell complement within a tubule. Cytokeratin was expressed in CIS tubules containing OCT4 positive, OCT4 negative or VASA positive cells. This suggests that the differentiation status of the Sertoli cells is not directly related to the differentiation status of the associated CIS/germ cells and that this is unlikely to explain the variation in differentiation status of Sertoli cells in different regions of the testis. However it was not determined whether the VASA positive cells within the tubules are CIS cells or differentiated germ cells, given that the present studies have shown that no single marker (including OCT4) is expressed by every CIS cell. Communication between Sertoli cells and CIS cells has been proposed to have a role in maintaining the latency period in CIS cells during childhood and that loss of communication between the Sertoli cells or between Sertoli cells and CIS cells may be important for subsequent proliferation and invasion of CIS cells in adulthood (Brehm et al., 2002). De-differentiation of Sertoli cells may be the cause of the loss of intercellular communication (Brehm et al., 2002). A loss of intercellular communication has also been described in CIS cells (Gondos et al., 1983) and this may also play a role in invasive progression.

3.5.4. Conclusion

This chapter has provided new insight into the phenotype of CIS cells and demonstrated that some germ cells with the histological characteristics of CIS cells do not appear to express 'classical' markers such as OCT4, whilst others do, resulting in various subpopulations of CIS cells. Using a panel of antibodies no single marker was found to be expressed in all CIS cells. Because of this heterogeneity of protein expression, distinguishing between CIS cells and differentiated germ cells within a tubule is difficult and requires determination of multiple markers. Proliferation occurs at a variable rate in the different CIS subpopulations, suggesting that some subpopulations may have an increased

tendency to invasive progression. No clear association was seen between the differentiation status of the Sertoli cells and their associated CIS cells. This chapter has emphasised the importance of correctly establishing the identity of cells within a CIS tubule to avoid inaccurate interpretation. The heterogeneity of protein expression in CIS cells in patients with TGCT could result from a variable cell of origin, or alternatively from subsequent differentiation of CIS cells. A comparable animal model of fetal testicular development may provide more insight into the origins and progression of CIS. The next chapter will examine the Common Marmoset monkey as such a model.

4 The Common Marmoset as a model for germ cell differentiation and proliferation

4.1. Introduction

4.1.1. The importance of testicular germ cell differentiation

Male fertility depends on the daily production of millions of sperm, and this is dependent upon the continuous, controlled proliferation of spermatogonia. The latter develop from relatively undifferentiated fetal germ cells (gonocytes), a process that is initiated in fetal life (Fukuda et al., 1975). If differentiation from a gonocyte to a spermatogonium and subsequent progression through meiosis does not occur, then this cell cannot contribute to spermatogenesis in adulthood. Failure of normal germ cell differentiation results in reduced fertility and may predispose to the development of TGCT (Rajpert-De Meyts, 2006). Studies relating to these conditions require an understanding of normal reproductive development and this understanding can be enhanced by identifying relevant animal models.

4.1.2. Current animal models of early germ cell development

Rodent studies have provided some insight into the maturation of the germ cell lineage in fetal/neonatal life (Wilhelm et al., 2007), but their usefulness is limited as there are some fundamental differences in the 'set-up' of spermatogenesis between rodents and humans. For example, the profile of gonadotrophin secretion is different in rodents when compared to humans. In humans there is a neonatal period of reproductive hormonal activity (neonatal testosterone rise or 'mini-puberty') (Mann and Fraser, 1996), followed by a period of 'childhood' quiescence (Kelnar et al., 2002) prior to the onset of puberty. This quiescent period does not occur in rodents (Plant, 2006). In addition, compared to the human, rodents exhibit a high efficiency of spermatogenesis compared to some primates (Millar et al., 2000) and humans (Clermont, 1963). The 'segmental' arrangement of spermatogenic

stages in the seminiferous tubules of rodents may be associated with more efficient spermatogenesis than the 'mixed' arrangement in the human. Differentiation of gonocytes into spermatogonia occurs during fetal life in both humans and rodents, however the process appears less synchronised in the human, with gonocytes still detectable in early postnatal life (Hoei-Hansen et al., 2005);(Hoei-Hansen et al., 2004). The persistence of gonocytes in the postnatal testis in humans may be important in the pathogenesis of TGCT (Rajpert-De Meyts et al., 2003) and this may partially explain why TGCT have never been observed in rodents. There are also differences in the organisation of germ cell proliferation between human and rodents. For example, rodents exhibit synchronised cessation of proliferation during the perinatal period (Ferrara et al., 2006), which is not apparent in the human (Honecker et al., 2004).

4.1.3. The marmoset as a potential animal model of germ cell development

Based on previous studies the Common Marmoset (*Callithrix jacchus*), a small New World primate, could potentially provide a better animal model for human germ cell development than rodents. The marmoset has many similarities to the human in terms of timing of testis development and function, in addition to the subsequent organisation and efficiency of spermatogenesis. This includes the neonatal period of reproductive hormonal activity (Kelnar et al., 2002). In adulthood, similar to the human, there is a 'mixed' organisation of spermatogenic stages within the tubule, in contrast to rodents and other primate models in which there is a 'segmental' organisation of the spermatogenic stages (Sharpe, 1994);(Weinbauer et al., 2001);(Millar et al., 2000). Additionally, the germ cell to Sertoli cell ratio is low in the marmoset, as in the human (Sharpe et al., 2000), indicating low efficiency of spermatogenesis when compared with rodents. These similarities led us to consider if the marmoset might also be a better model than rodents in terms of perinatal testis development and in particular germ cell differentiation and proliferation.

4.2. Chapter aims

This chapter aimed to investigate testicular germ cell development in the fetal and postnatal marmoset, with particular emphasis on differentiation and proliferation in the fetal and early postnatal period. The chapter also aimed to compare and contrast the findings with the human and rodent.

4.3. Materials and Methods

4.3.1. Marmoset tissue collection

Marmoset testes from fetal (11 weeks, n=1; 14 weeks, n=2; 15 weeks, n=1; 16 weeks, n=2; 17 weeks, n=2; 20 weeks, n=2) and postnatal (aged 1 day, 2, 6, 22, 35 or 48 weeks, adult; n=3 for each age) animals were obtained as described in 2.4.1.

4.3.2. Human fetal testis tissue collection

Human fetal testis tissue was obtained as described in Section 2.1.1. First trimester testes (8-10 weeks, n=4), 2nd trimester (14-19 weeks, n=9), third trimester (27-31 weeks, n=2) and postnatal (4 weeks – 11 months, n=4) testes were used for these experiments.

4.3.3. Rat testis tissue collection

Testes were obtained from e15.5 and e19.5 rat fetuses as described in 2.4.3.

4.3.4. Haematoxylin and eosin staining

The method for haematoxylin and eosin staining is described in section 2.9.

4.3.5. Immunohistochemistry

Single immunohistochemistry was performed with DAB detection as described in section 2.10. The primary antibodies used for these experiments are listed in Table 2.1. Double staining was performed with DAB and fast blue detection as outlined in

section 2.10.3. Details of conditions for double staining experiments can be found in Table 2.2.

4.3.6. Immunofluorescence

Double immunofluorescence was performed as described in section 2.11.1. The primary and secondary antibodies, and detection labels used for these experiments are listed in Table 2.3.

4.3.7. Quantification of germ cell subpopulations and proliferation

Quantification of proliferating germ cells was calculated for marmoset and human testes as described in section 2.12.1. For quantification in fetal samples immunohistochemistry for Ki67 was performed as described in section 2.10. The fetal germ cells were identified based on morphology and position within the seminiferous cords, whilst postnatal germ cells were identified using VASA/Ki67 co-staining as described in 2.10.3. Sections were counted independently by two observers (Myself and Dr Gillian Cowan) to ensure accuracy of germ cell identification. Excellent agreement between the two observers was found. The proliferation index was calculated by dividing the number of Ki67-positive germ cells by the total number of germ cells assessed in thirty randomly selected fields.

4.3.8. Western blotting

Fetal testis tissue was obtained for Western analysis as described in section 2.13.1. Details of the protein extraction, quantification and Western blotting procedure can be found in section 2.13. Details of the primary antibodies used are found in Table 2.5.

4.4. Results

4.4.1. The reproductive tract of the fetal male marmoset monkey

The general appearance of the marmoset male fetal reproductive tract can be seen in Figure 4.1. During fetal life the testes are intra-abdominal and are situated inferior to the kidney. The adrenal glands in the fetal marmoset are large relative to the size of the kidney.

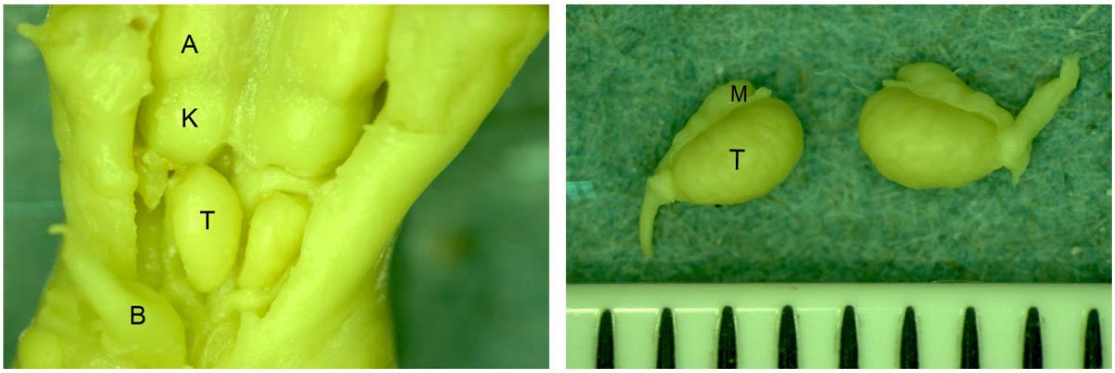

Figure 4.1. General structure of the 98 day male fetal marmoset reproductive tract. Left panel shows the testis (T) position within the abdomen, with the bladder (B) moved laterally to expose the testes. Note the large size of the fetal adrenal gland (A) relative to the kidney (K). The right panel shows the gross structure and size of the testis (T) and its associated mesonephros (M). Note that the markings on the ruler in the right hand panel are 1mm apart.

4.4.2. Localisation of cell types within the fetal marmoset testis

Testes obtained from fetal marmosets between 11 weeks gestation and term all contained well defined testicular cords surrounded by an interstitial compartment (Fig. 4.2). The seminiferous cords were composed mostly of Sertoli cells with a few germ cells identified within each cord.

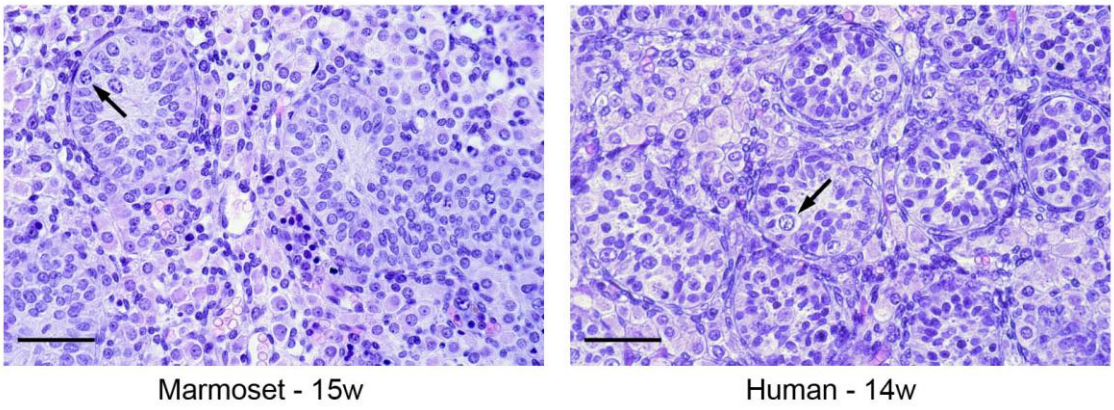

Figure 4.2. General structure and organisation of the fetal testis in the human and marmoset. Germ cells (arrows) are identified within the cords. Scale bar 50μm. w=weeks.

Sertoli cells were identified both histologically (small, irregular shaped nuclei) and by the cytoplasmic localisation of anti-müllerian hormone (AMH), which was expressed in all the testes examined (Fig. 4.3). Germ cells were identified within the cords, because the nuclei had a larger diameter than the Sertoli cells, they expressed one or more germ cell markers (see below) and they lacked expression of AMH (Fig. 4.3). Within the interstitium, steroidogenically active Leydig cells were identified by expression of 3β-HSD (Fig. 4.3).

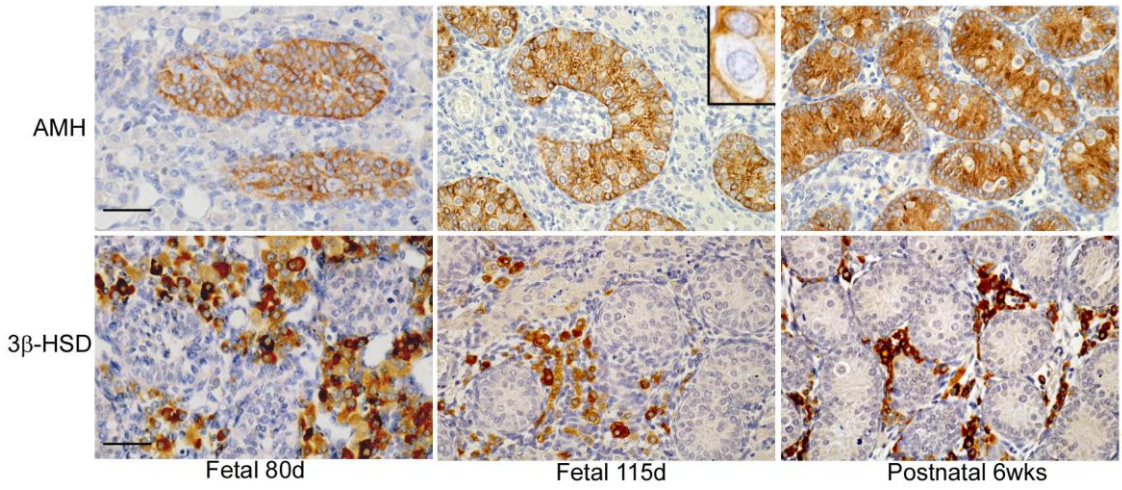

Figure 4.3. Localisation of cell types within the fetal and early postnatal marmoset testis. Immunoexpression of AMH in Sertoli cells within seminiferous cords (upper panels) and 3β-HSD in Leydig cells in the interstitium (lower panels). In the top panels, the clear, unstained cytoplasm surrounds the nuclei of germ cells within seminiferous cords (inset). Scale bar 50μm. wks=weeks, d=days.

Cytokeratins detected with a pan-cytokeratin antibody were detected in the cytoplasm of Sertoli cells in fetal testes of both marmoset and human (Fig. 4.4). The peritubular myoid cells of both species were immunopositive for nuclear androgen receptor (Fig. 4.4). In addition there is expression of androgen receptor in a proportion of the interstitial cells, whilst Sertoli cells were immunonegative.

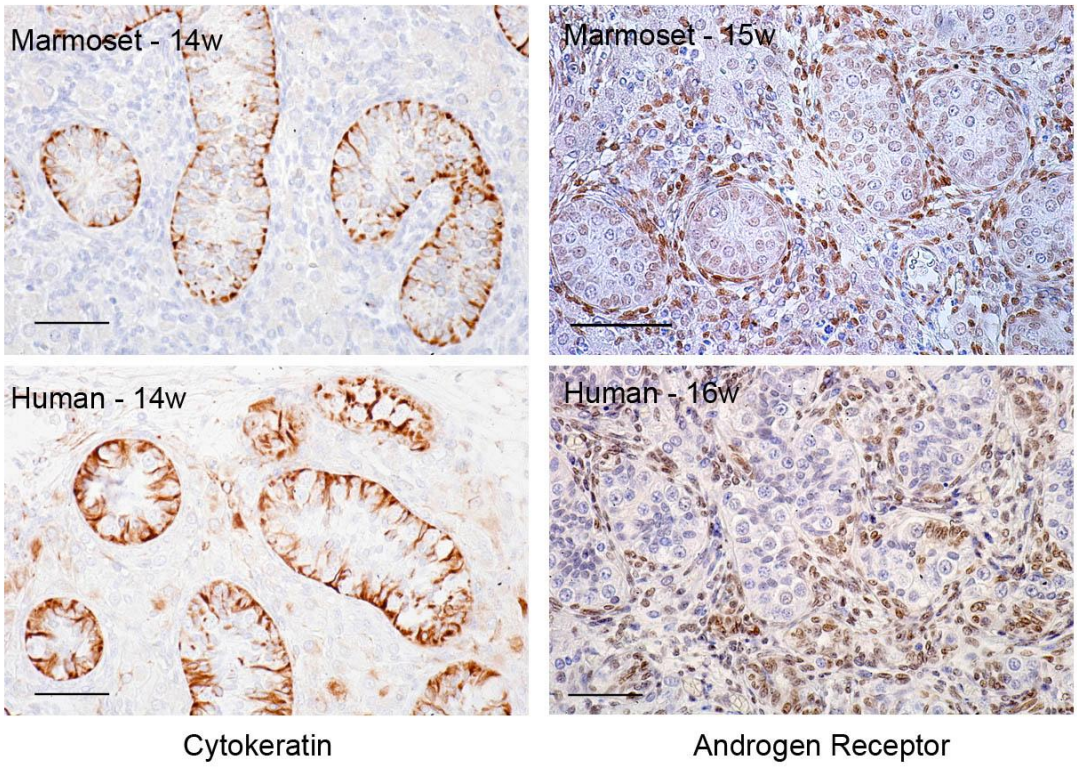

Figure 4.4. Immunoexpression of pan-cytokeratin and androgen receptor in fetal marmoset and fetal human testis. Pan-cytokeratin (left panels) is expressed in Sertoli cells and androgen receptor (right panels) is expressed in peritubular myoid and some interstitial cells. Scale bar 50μm. w=weeks.

4.4.3. Germ cell differentiation in the marmoset and human

4.4.3.1. Expression of markers of pluripotency and early germ cell differentiation in fetal life

In the marmoset expression of AP-2γ, NANOG and OCT4 was germ cell specific and was detected in a decreasing proportion of the total germ cell population during progression through fetal life into the early postnatal period (Fig. 4.5). All three proteins were expressed in the majority of germ cells at 14 weeks' gestation, but by the end of pregnancy the proteins were only immunolocalised to a small subset of germ cells. For NANOG, germ cell expression ceased between 2 and 6 weeks of postnatal age, whilst OCT4 and AP-2γ expression persisted in a few, scattered germ cells at 6 weeks postnatally (Fig. 4.5).

A similar expression pattern was demonstrated in fetal human testes. OCT4 (Fig. 4.5), NANOG and AP-2γ were detected in most testicular germ cells of first trimester fetuses (9 weeks) but the proportion of immunopositive germ cells decreased as gestation progressed. A few OCT4 positive germ cells were still present at 4 weeks postnatally (Fig. 4.5) and occasionally at 6 months after birth (Fig. 4.6) and similar results were obtained for AP-2γ. NANOG was detected in occasional germ cells at 4 weeks of postnatal age but not in tissue from older individuals (Fig. 4.5).

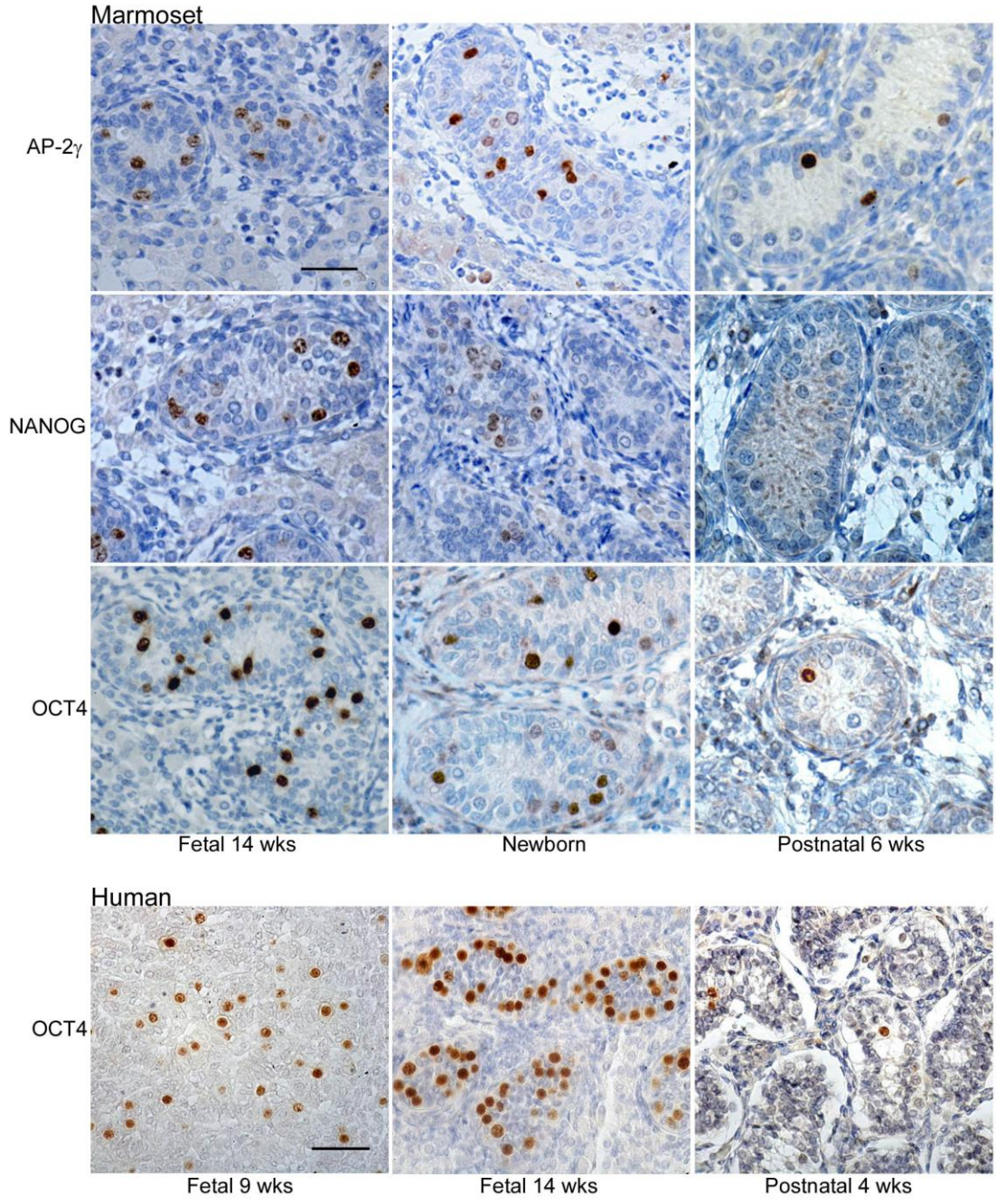

Figure 4.5. Immunoexpression of gonocyte markers (AP-2γ, NANOG, OCT4) in the marmoset testis and comparison with the human. Note that the proportion of germ cells expressing these markers decreases with increasing age and the similar pattern of OCT4 expression in the marmoset compared to the human. Scale bar 50μm. wks=weeks.

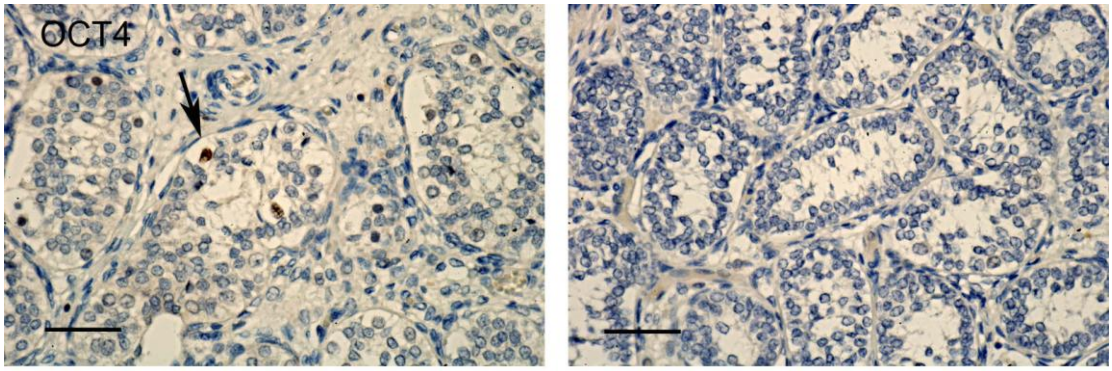

Figure 4.6. Persistence of OCT4 expression in occasional germ cells in a 6 month infant human testes. Note OCT4 (arrow) expression in the 6 month testis, but no expression in an 11 month old infant. Scale bar 50μm. m=months.

4.4.3.2. Expression of proteins associated with germ cell differentiation in perinatal life

In the marmoset during fetal life there was an increase in the proportion of germ cells that were immunopositive for MAGE-A4 and VASA as gestation progressed (Fig. 4.7). VASA or MAGE-A4 were expressed within the cytoplasm of a small proportion of germ cells by 15 weeks. By birth the majority of germ cells were positive for VASA and similar results were obtained for MAGE-A4. By 6 weeks of postnatal age only the occasional germ cell remained immunonegative for these proteins (Fig. 4.7).

In the human, VASA immunopositive germ cells were first detected in second trimester testes, with an increasing proportion of germ cells expressing VASA in the third trimester (Fig. 4.7). In the early postnatal period almost every germ cell expressed VASA. A similar pattern of expression was also found for MAGE-A4 (Fig. 4.7).

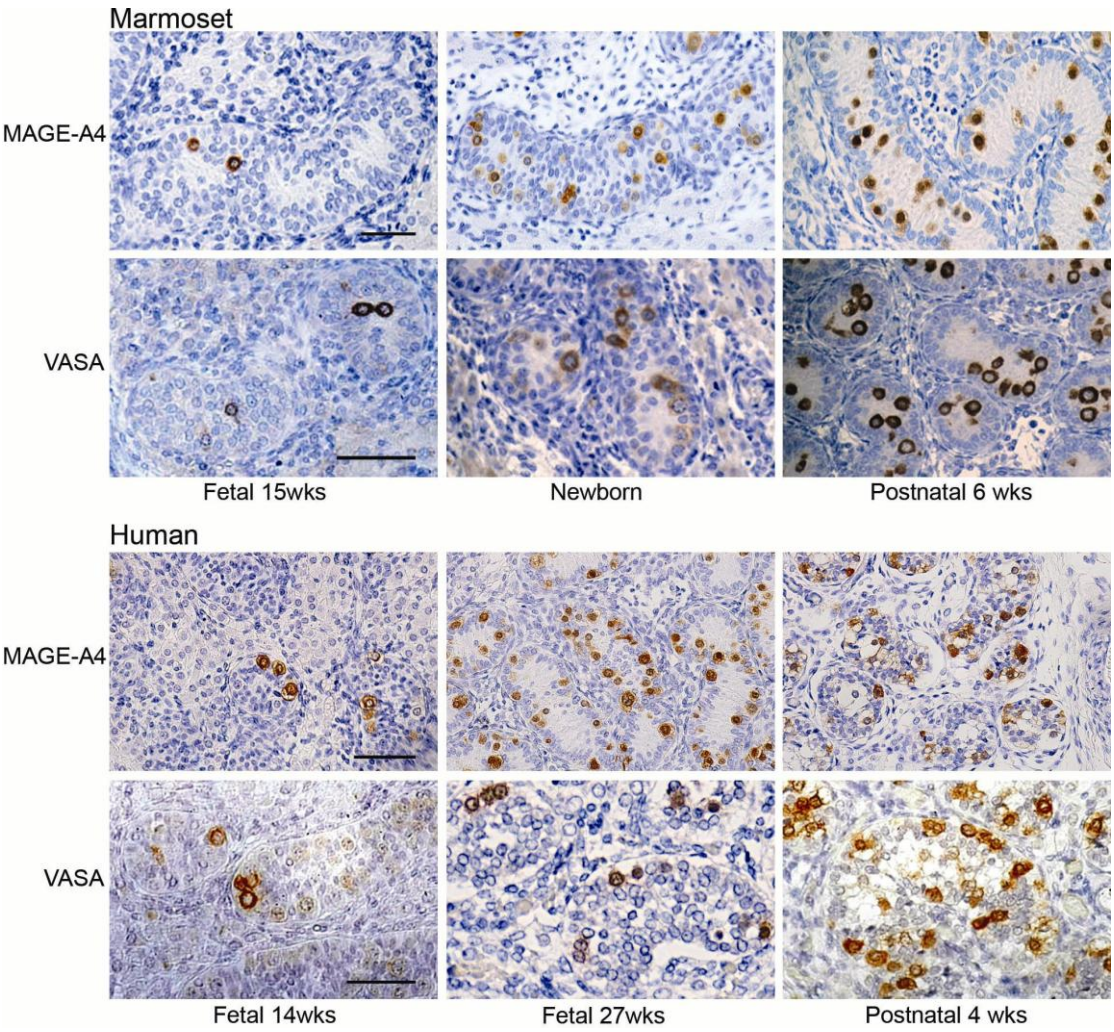

Figure 4.7. Immunoexpression of MAGE-A4 and VASA in the marmoset testis and comparison with the human. Note the increasing proportion of germ cells expressing these markers in the marmoset and human. wks=weeks Scale bar 50μm.

The specificity of the antibody for VASA was tested by Western blotting of fetal human and postnatal marmoset testes. For VASA a single band at the expected molecular weight of 67 KDa was obtained in fetal human and newborn marmoset testes (Fig. 4.8).

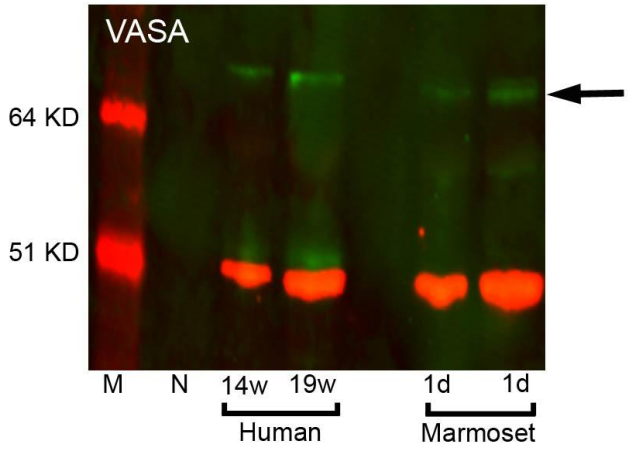

Figure 4.8. Western blot for VASA in fetal human and neonatal marmoset testes. β-tubulin (51 KDa) was used as a loading control. Arrows indicate the band corresponding to the VASA (green) protein at 67KDa. M = molecular weight marker, N = negative control, w=weeks, d=days.

4.4.3.3. Co-localisation of germ cell markers in the fetal marmoset, human, and rat

Co-localisation studies were used to investigate the relationship between germ cells expressing proteins previously localised to human gonocytes (OCT4, NANOG, AP-2γ) and those expressing VASA, MAGE-A4 or NANOS-1. These investigations revealed a distinct and only partially overlapping pattern of expression in germ cell subpopulations in both the marmoset and human. For example, co-localisation of AP-2γ with VASA confirmed our earlier findings of a decreasing proportion of AP-2γ positive cells and an increasing proportion of VASA positive cells with increasing age (Fig. 4.9). In addition a small proportion of cells were positive for both proteins at the same time. Notably, within a single testicular cross section mixed populations of germ cells were detected within individual cords, such that a single cord could contain AP-2γ^+/VASA$^-$, AP-2γ^+/VASA$^+$ or AP-2γ^-/VASA$^+$ germ cells; this pattern was equally evident in the marmoset and human. The existence of seminiferous cords containing a germ cell cohort that was predominantly AP-2γ^+/VASA$^-$ and those that were mostly AP-2γ^-/VASA$^+$ appeared to be separated by a period of several months from fetal into early postnatal life, in both the marmoset and the human. Germ cells

were not synchronised in their expression of these proteins between or within the seminiferous cords of an individual fetus/neonate and there was also some variation in the timing of expression between individuals of the same age (Fig. 4.9).

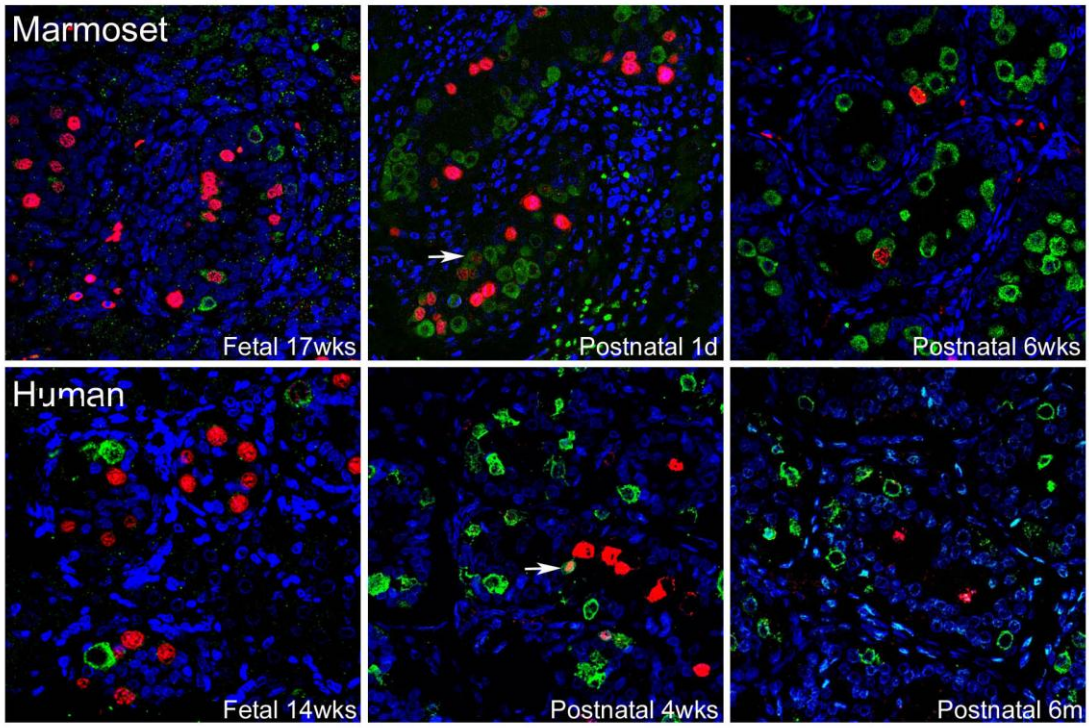

Figure 4.9. Expression of AP-2γ and VASA in human and marmoset testis during fetal and early postnatal life. Distinction between undifferentiated germ cells expressing AP-2γ (red, nuclear localisation) and differentiated germ cells expressing VASA (green, cytoplasmic localisation) can be seen in both human and marmoset. Note that all germ cells are visualised with this combination of markers. A small proportion of germ cells co-expressed both markers (arrows). Sections counterstained with TO-PRO-3 (blue, nuclear). wks=weeks, d=days, m=months.

In the marmoset and human a similar expression profile was demonstrated for OCT4 and VASA (Fig. 4.10), with co-localisation of both proteins in a small proportion of germ cells, resulting in three different subpopulations, namely $OCT4^+/VASA^-$, $OCT4^+/VASA^+$ and $OCT4^-/VASA^+$. However in the fetal rat, there was no evidence for an $OCT4^+/VASA^-$ population of germ cells. Co-localisation of OCT4 and VASA was detected in all germ cells at e15.5. By e19.5, no germ cells expressed

OCT4, but all continued to express VASA. The loss of OCT4 immunoexpression appeared to be synchronous in all germ cells in every cord over this relatively narrow time period (Fig. 4.10).

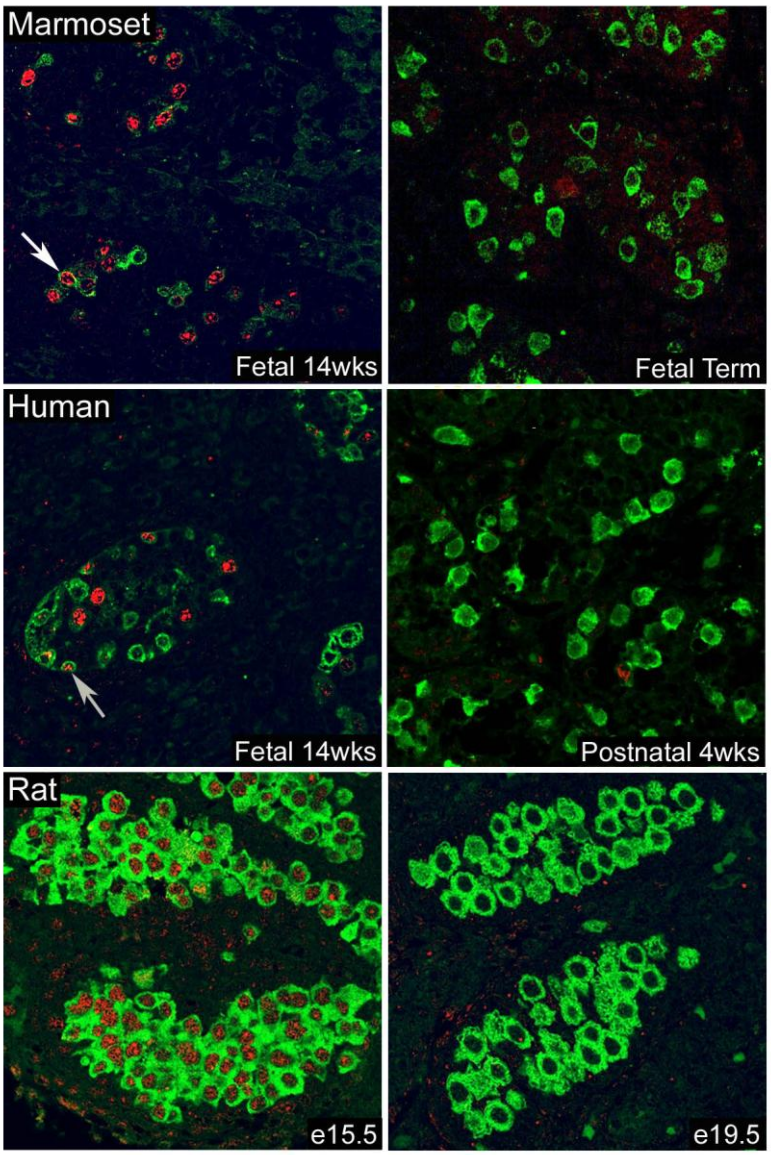

Figure 4.10. Comparison of perinatal germ cell differentiation in the marmoset, human and rat. Expression of protein markers of undifferentiated (OCT4: red, nuclear localisation) and differentiated (VASA: green, cytoplasmic localisation) germ cells. Note that in seminiferous cords in both the marmoset and human at each age studied, there is a mixed population of germ cells in the human and marmoset, including occasional cells in which the markers were co-localised (arrows). In the rat OCT4 is co-expressed with VASA in all germ cells at e15.5 but is no longer detected within any of the germ cells at e19.5. wks=weeks.

4.4.3.4. Expression of NANOS-1 in the fetal and postnatal marmoset and comparison with the human

In the marmoset at 11 weeks' gestation none of the germ cells expressed NANOS-1 (Fig. 4.11). Increasing proportions of germ cells expressed NANOS-1 in the nucleus during the final third of gestation and by early postnatal life most of the germ cells contained NANOS-1, with localisation of the protein to both the nucleus and peri-nuclear cytoplasm (Fig. 4.11). Human testes were immunonegative for NANOS-1 in the first trimester, but this protein was expressed in the nucleus of germ cells from the second trimester onwards with an increasing proportion of germ cells expressing this protein as gestation progressed. In postnatal life, NANOS-1 was also detected in the peri-nuclear cytoplasm of germ cells. Germ cells immunopositive for NANOS-1 also expressed VASA (Fig. 4.11, a). AP-2γ and NANOS-1 were not co-localised (Fig. 4.11, b), which was in contrast to results obtained when co-staining for AP-2γ and VASA was performed (Fig. 4.9), when a proportion of germ cells were found to be immunopositive for both proteins. This suggests that within an individual cell, expression of NANOS-1 begins shortly after VASA expression begins.

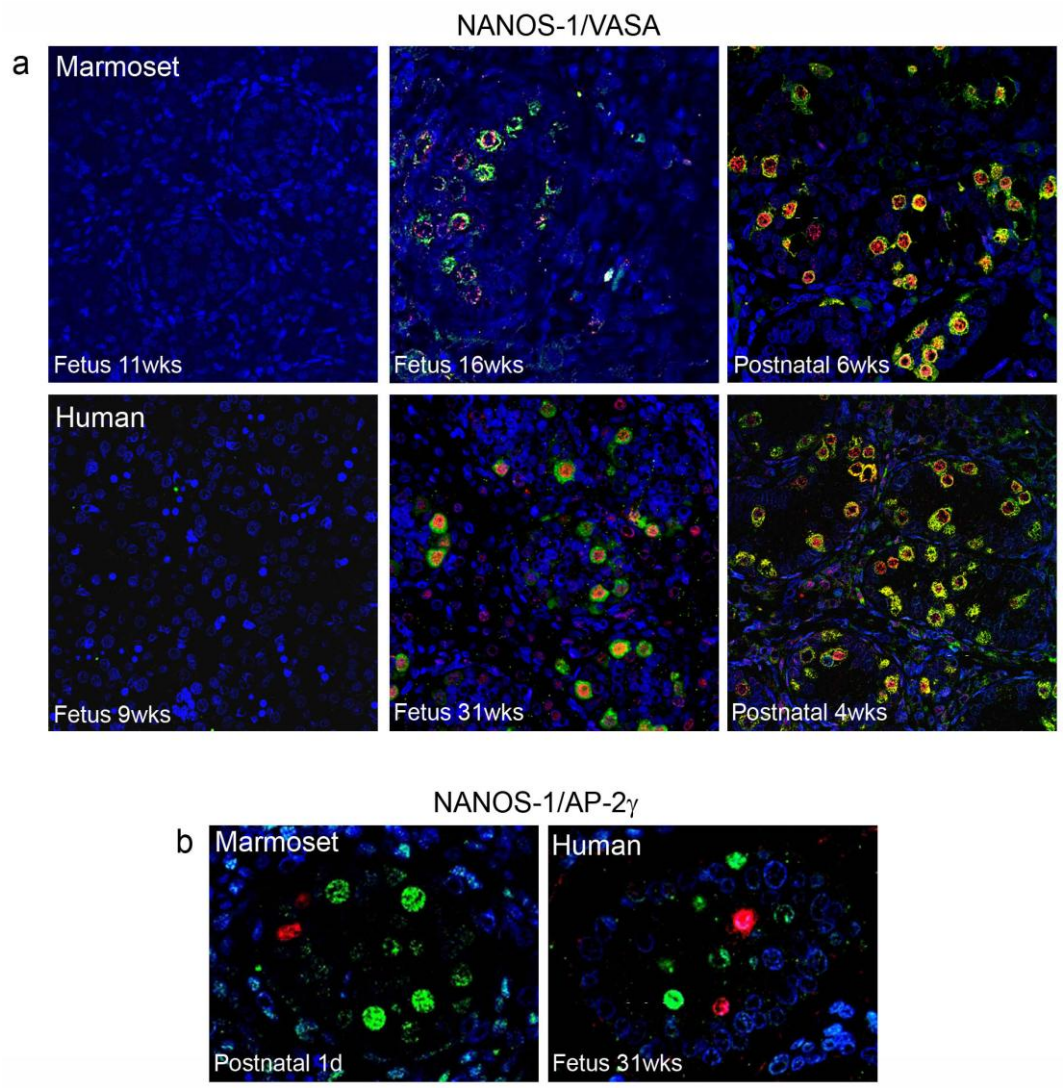

Figure 4.11. Expression of NANOS-1 with VASA and AP-2γ in human and marmoset testes during fetal and early postnatal life. a) Comparison of the immunoexpression of NANOS-1 (red, nuclear and cytoplasmic localisation) and VASA (green, cytoplasmic localisation) in germ cells in the testis during fetal and postnatal life in the marmoset (upper panels) and human (lower panels) testis. The proteins are expressed in the final third of gestation in the marmoset and in the second trimester in the human testis. In postnatal life co-localisation in the peri-nuclear region is seen (yellow) in most germ cells. b) Immunoexpression of NANOS-1 (green, nuclear localisation) and AP-2γ (red, nuclear localisation) in the marmoset and human testis for comparison with VASA/AP-2γ expression (Fig. 4.9). NANOS-1 is expressed in most of the germ cells in the neonatal marmoset (left panel) and 31 week gestation human testis (right panel), with the remainder expressing AP-2γ. However, in contrast to VASA/AP-2γ (Fig. 4.9), no germ cells co-localised NANOS-1 and AP-2γ. All sections counterstained with TO-PRO-3 (blue). wks=weeks, d=days.

4.4.3.5. Expression of KIT in the germ cells of the marmoset testis from fetal to adult life

KIT is expressed in gonocytes of the fetal marmoset testis (Fig. 4.12). The proportion of cells expressing KIT decreases as the cells differentiate from gonocyte to spermatogonia as described for OCT4, AP-2γ and NANOG (Fig. 4.5). Co-staining of MAGE-A4 and KIT demonstrated that the KIT stained gonocytes were distinct from the MAGE-A4 population. The KIT positive gonocytes are located throughout the tubules and many are found in a central position within these tubules. In the neonatal and early postnatal testis rare KIT^{+}/$MAGE\text{-}A4^{-}$ gonocytes persist but these cells are absent after 6 weeks of postnatal age. In the juvenile testis after the gonocytes have differentiated, KIT expression occurs again in a subset of spermatogonia (Fig. 4.12). These spermatogonia are located on the basement membrane and in contrast to the gonocytes these cells also express the spermatogonial marker MAGE-A4 (Fig. 4.12).

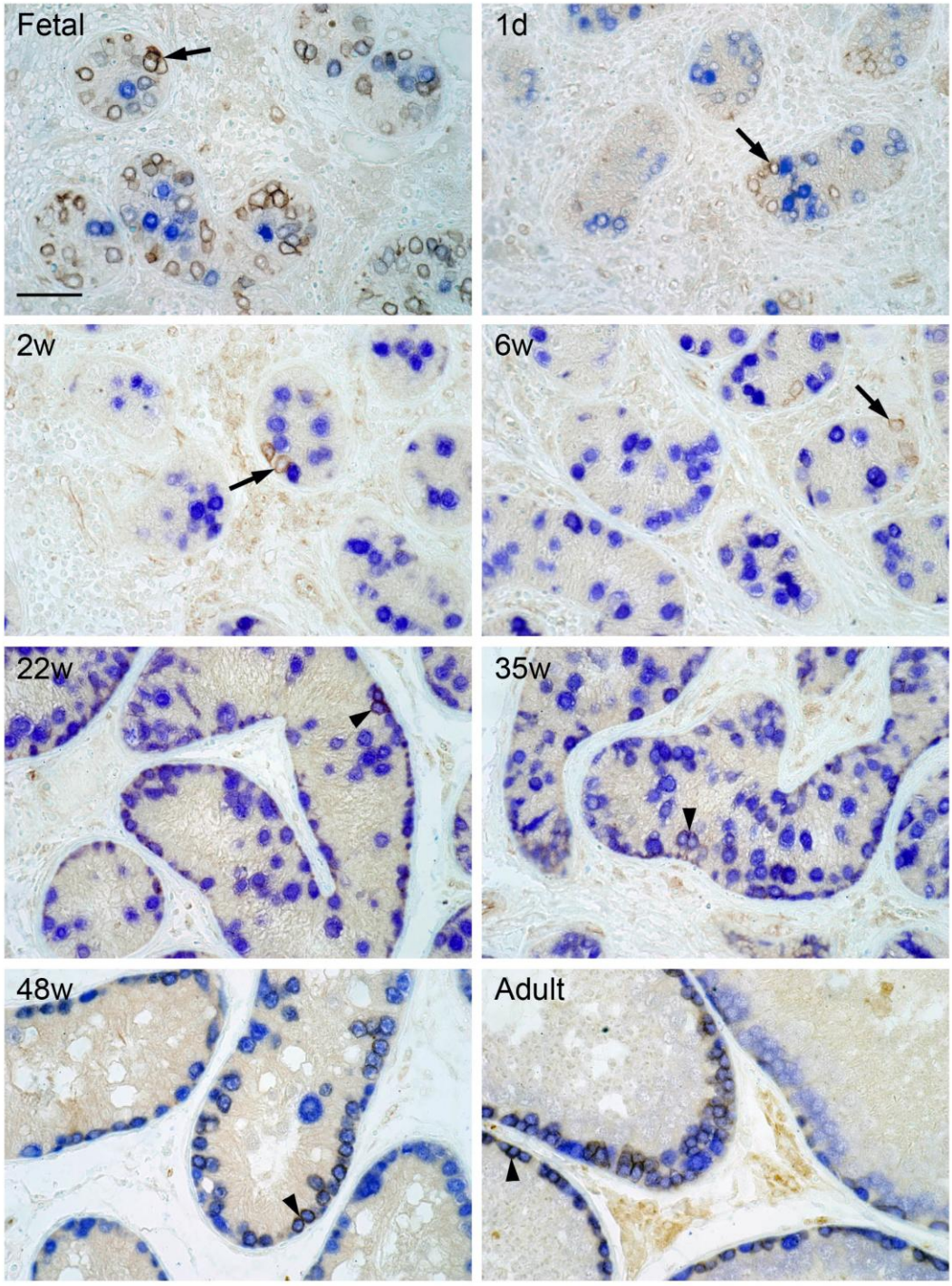

Figure 4.12. Expression of KIT and MAGE-A4 in fetal and postnatal germ cells of the marmoset testis. KIT (brown) is localised on the membrane of gonocytes (arrows), whilst later in postnatal life and into adulthood KIT can be detected in a subset of spermatogonia. MAGE-A4 is expressed in spermatogonia that are also immunopositive for KIT (arrowheads), but is not expressed in the gonocytes. w=weeks Scale bar = 50μm.

4.4.3.6. Germ cell differentiation in the pre-pubertal and adult marmoset testis

Following the neonatal period the marmoset enters infancy, then progresses through pre-puberty, puberty and adulthood. By the end of infancy the remaining gonocytes have differentiated into spermatogonia, which line the basement membrane. Sertoli cells separate these germ cells from the luminal surface of the tubule. As described previously, these spermatogonia are not immunopositive for pluripotency markers such as OCT4 and NANOG. Puberty commences at approximately one year in the marmoset, when the process of spermatogenesis begins. Expression of proteins such as VASA, MAGE-A4 and NANOS-1 within the germ cell population continues throughout this period (Fig. 4.13). VASA is expressed in all germ cell types from the end of the neonatal period and into puberty and adulthood. Immunoexpression of VASA is intense in the spermatogonia, late spermatocytes and spermatids, but is weak in the early spermatocytes. VASA can be detected in the cytoplasm of these cells. The pattern of expression of NANOS-1 is similar to that of VASA but there is less staining in the spermatogonia than with VASA, and NANOS-1 tends to be present in the nucleus and perinuclear cytoplasm. MAGE-A4 is expressed of spermatogonia and early spermatocytes with less intense staining in the later spermatocytes and spermatids. This staining may be detected in either the cytoplasm or nucleus (Fig. 4.13)

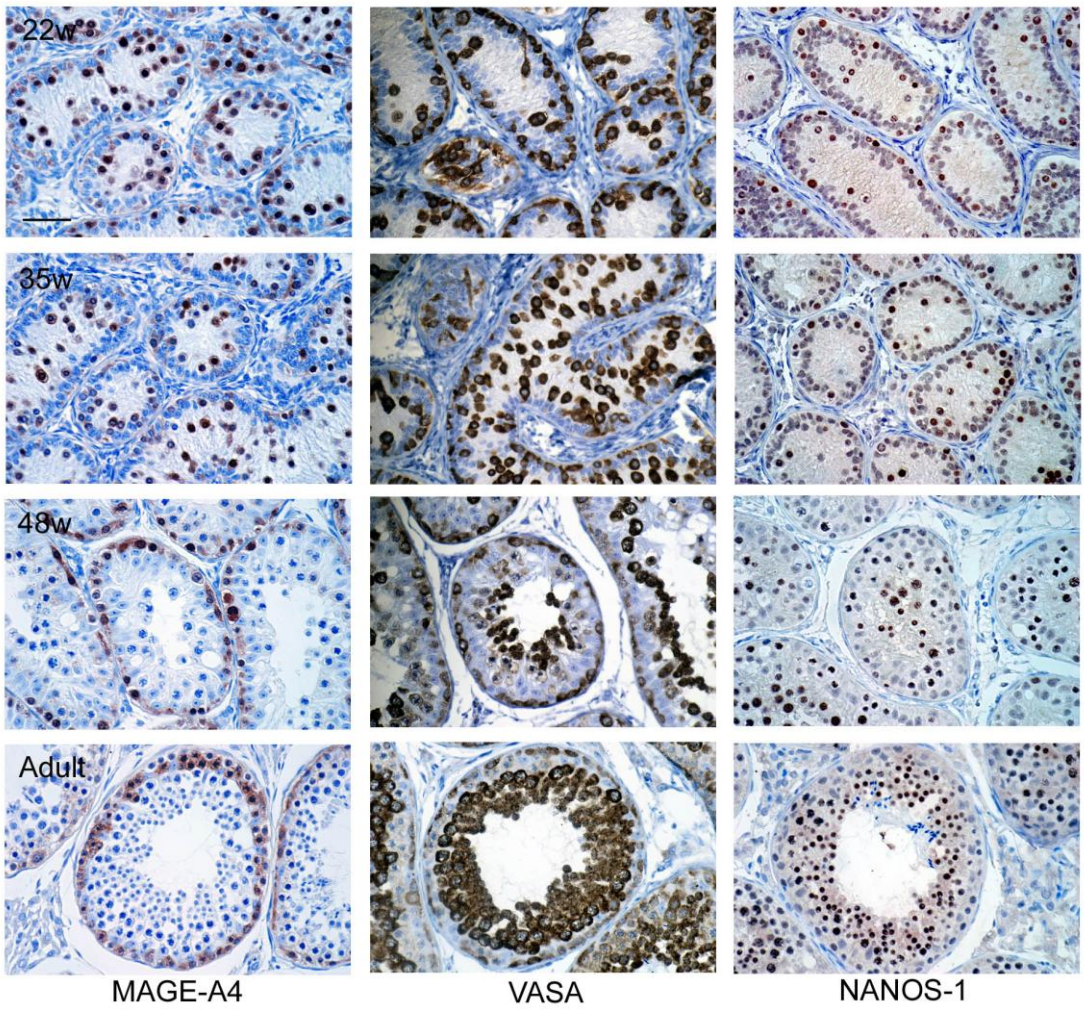

Figure 4.13. MAGE-A4, VASA and NANOS-1 expression in postnatal germ cells of the marmoset. Expression in pre-pubertal (22,35 weeks), pubertal (48 weeks) and adult marmoset. MAGE-A4 is mainly expressed in spermatogonia and early spermatocytes, whilst VASA and NANOS-1 are expressed in the majority of germ. w=weeks Scale bar = 50μm.

4.4.4. Germ cell proliferation in the marmoset and human

4.4.4.1. Germ cell proliferation in the perinatal marmoset, human and rat testis

Immunoexpression of Ki67 was detected in the marmoset testis at all fetal ages investigated from 11 weeks' gestation until birth, with a significant reduction in the number of Ki67 positive germ cells during weeks 15-16 (Fig. 4.14). Ki67 positive germ cells were also found in postnatal life, although they were less frequent than

during the majority of fetal life (Fig. 4.14). In the human fetal testis, germ cell proliferation occurred in a relatively high proportion of germ cells in the first and second trimester, similar to that seen in the fetal marmoset (Fig. 4.14). The proportion of germ cells proliferating in the first trimester was significantly higher than in the second trimester ($p<0.01$). Unfortunately poor preservation of third trimester tissue prevented us from accurate determination of the proliferation index.

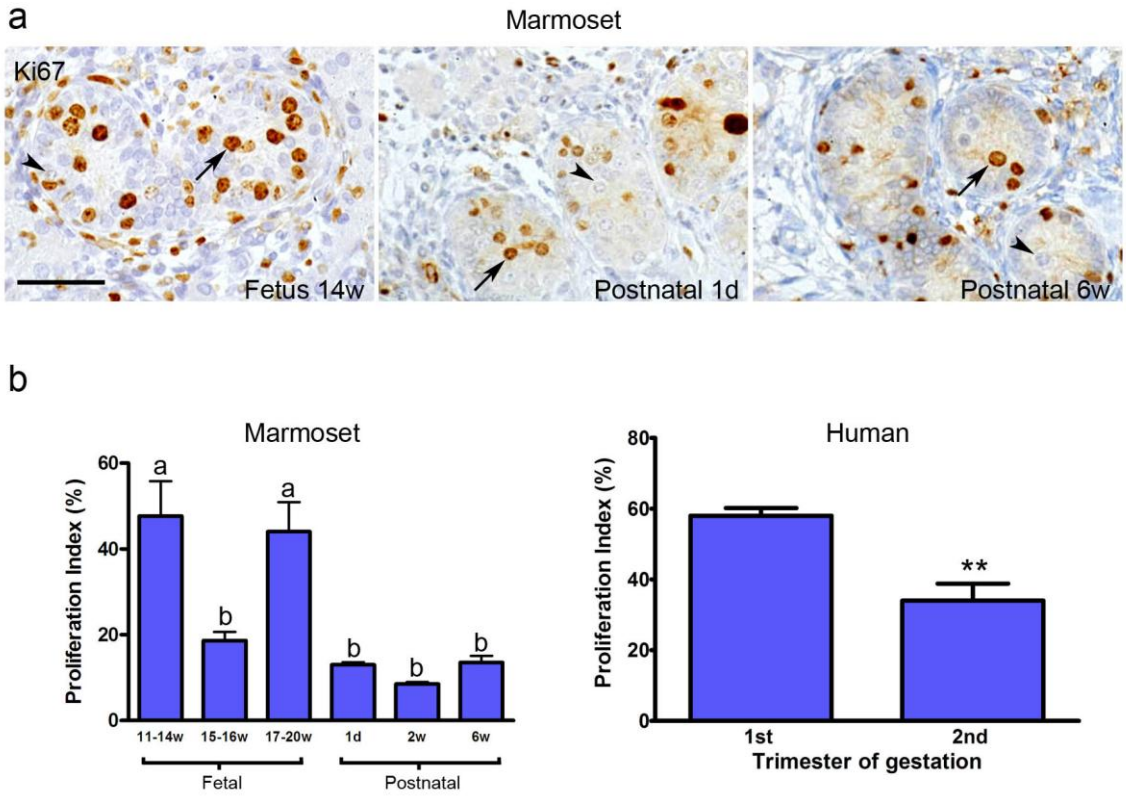

Figure 4.14. Germ cell proliferation in the human and marmoset testis in fetal and early postnatal life. a) Germ cell proliferation in the marmoset testis in fetal and early postnatal life determined by immunoexpression of Ki67 (brown). Germ cell proliferation (arrows) occurs in a proportion of germ cells throughout fetal and postnatal life in the marmoset. At all ages a variable proportion of germ cells are not proliferating (arrowheads). Note that some Sertoli cells are also proliferating in the sections. Scale bar = 50μm. b) Proliferation index for germ cells in fetal and postnatal marmoset (left panel) and first and second trimester human fetal testes (right panel). Groups labelled with an 'a' are significantly different from those marked with a 'b'. ** $p<0.01$, compared with 1st trimester values. Means ± sem ($n\geq3$ for each age). w=weeks, d=days.

In both the marmoset and human, proliferation occurred in a proportion of both undifferentiated (AP-2γ positive, gonocytes) and differentiated (VASA positive spermatogonia) germ cells (Fig. 4.15).

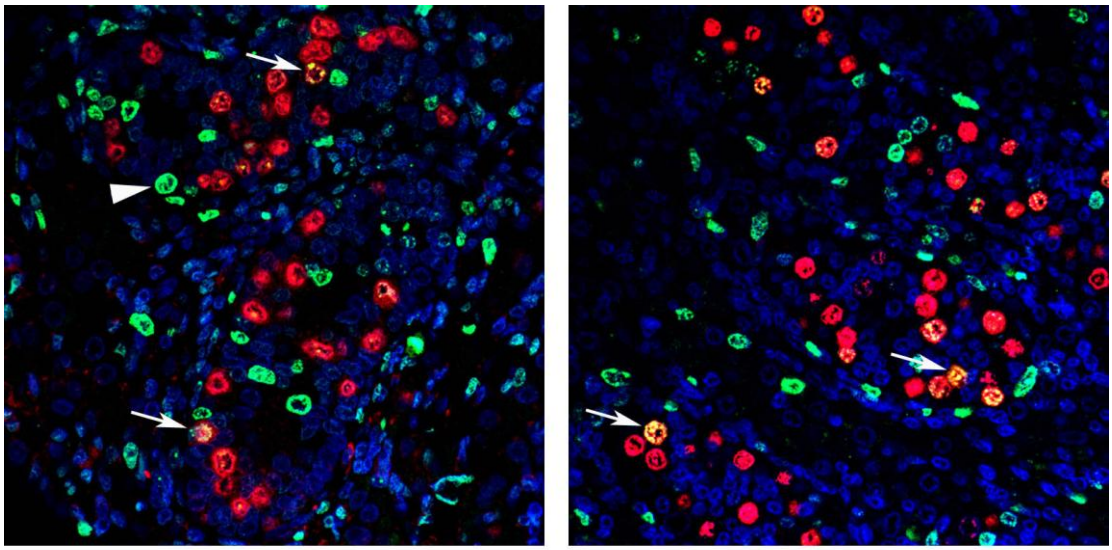

Figure 4.15. Proliferation of gonocytes in the human and marmoset fetal testis. Immunoexpression of Ki67 (green) and AP-2γ (red) in fetal marmoset (left panel) and fetal human (right panel) testis. Germ cell proliferation occurs in a proportion of undifferentiated germ cells (arrows). Note that there are also proliferating germ cells that do not express AP-2γ (arrowhead), which would be expected to express VASA. w=weeks.

In contrast to the situation in the human and marmoset, germ cell proliferation in the fetal rat testis ceases completely in late gestation (Fig. 4.16). A large proportion of germ cells are proliferating at e15.5, but by e19.5 the germ cells have ceased proliferation and remain quiescent until postnatal life.

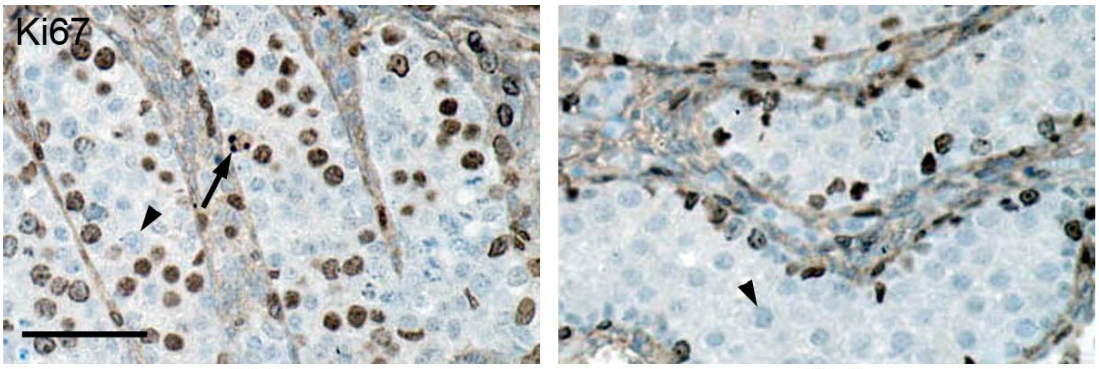

Figure 4.16. Germ cell proliferation in the fetal rat testis. Germ cell proliferation (Ki67; arrows) occurs in the majority of germ cells at e15.5 but has ceased completely by e19.5 when all germ cells are negative for Ki67 (arrowhead). Note that at both ages, some Sertoli cells (peripherally located) are immunopositive for Ki67. Scale bar 50μm.

4.4.4.2. Germ cell proliferation in the pre-pubertal and adult marmoset testis

In the marmoset, proliferation was noted in a proportion of germ cells at all ages studied when sections were co-stained with Ki67 and VASA (Fig. 4.17). Proliferating germ cells were more frequent in infancy (2,6 weeks) and puberty/post puberty (48 weeks, adult), whilst there were fewer co-stained cells during the childhood phase (22, 35 weeks).

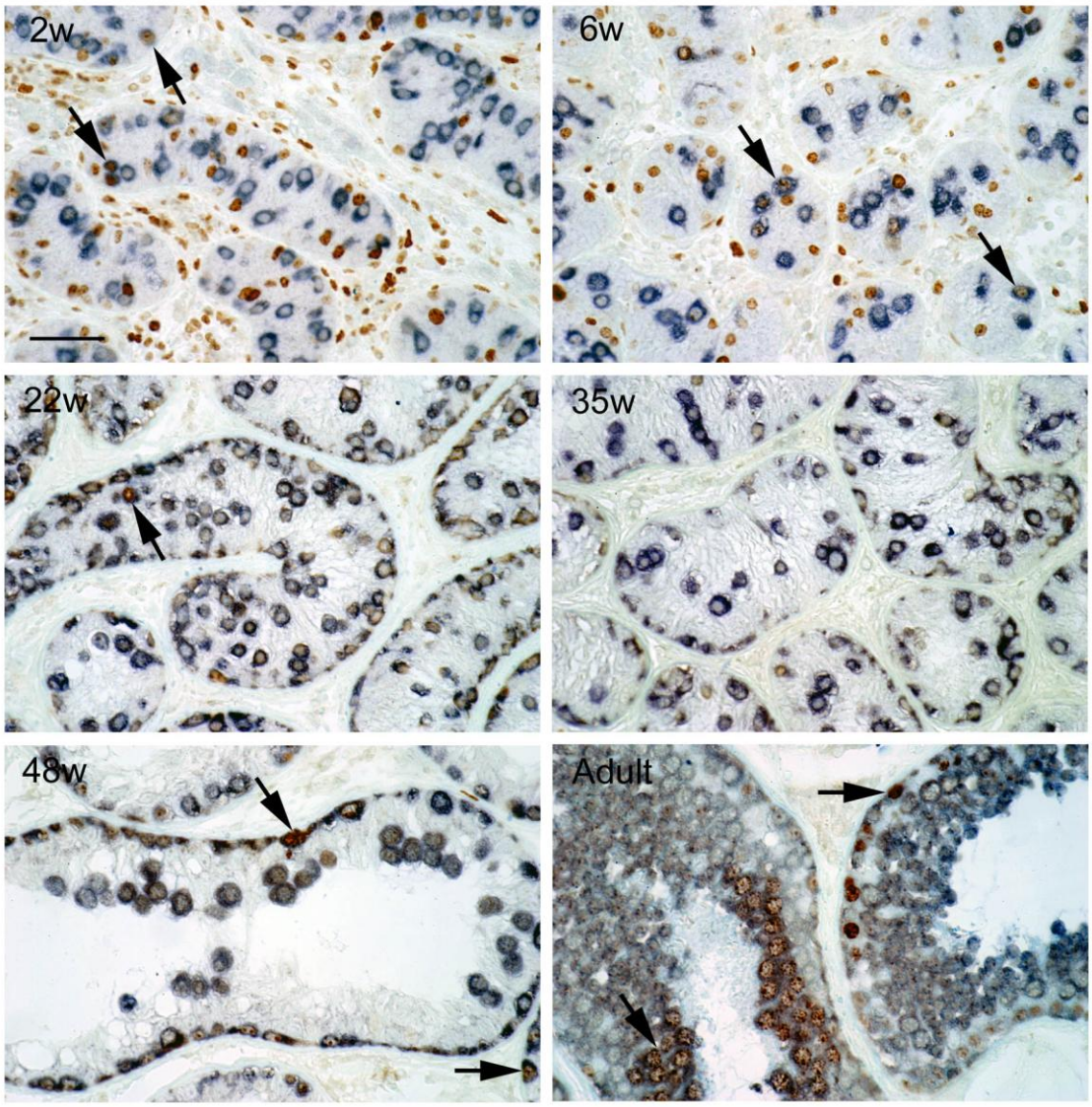

Figure 4.17. Proliferation of germ cells in the postnatal marmoset testis. VASA (blue; cytoplasmic) and Ki67 (brown; nuclear) co-staining in postnatal marmoset testes from birth to adulthood for use in quantification studies below. Co-stained cells (arrows) represent proliferating germ cells. w=weeks. Scale bar = 50μm.

The variation in the proliferation index at different ages was quantified (Fig. 4.18) and it demonstrated a significant decrease in germ cell proliferation during the childhood phase (22,35 weeks), compared to the majority of neonatal and pubertal life.

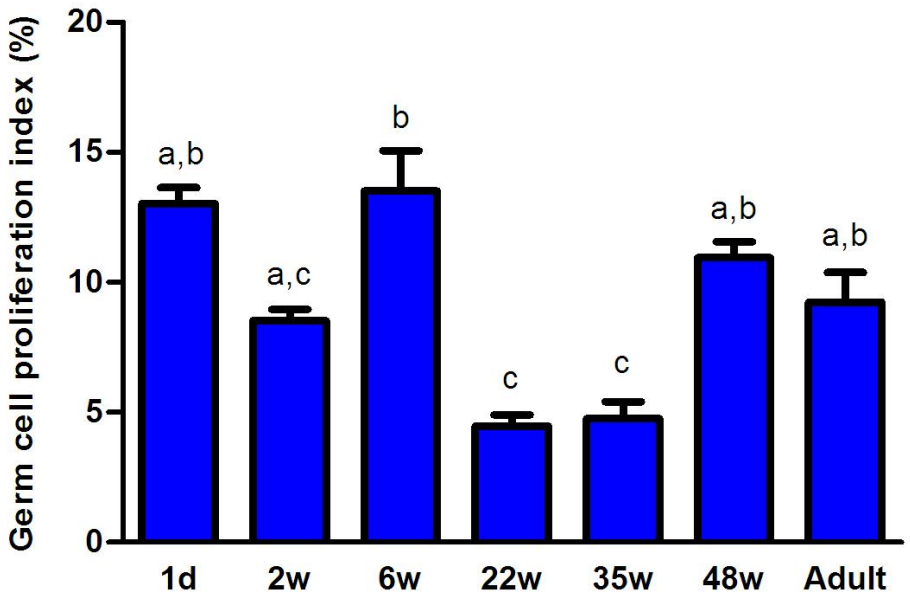

Figure 4.18. Quantification of germ cell proliferation in the marmoset testis from birth to adulthood. Mean ± sem (n≥3 for each age). Bars with letters in common are not significantly different. w=weeks, d=days.

4.5. Discussion

4.5.1. The structure and morphology of the fetal marmoset testis is similar to the human

Despite the similarities that exist between the marmoset and the human in terms of reproductive biology, little is known about germ cell differentiation and proliferation in the marmoset. In particular the development of fetal germ cells in the marmoset testis has not been described in detail. In fetal life the testes in both the human (Heyns and Hutson, 1995) and marmoset are intra-abdominal. We have demonstrated that seminiferous cords are present in the testes of the marmoset at 11 weeks' gestation and that there is a similar organisation of the cords compared to the human. The general organisation of the cell types within the fetal marmoset testis was comparable to that described in the human fetal testis (Wartenberg,

1981);(Gaskell et al., 2004). Identification of the particular cell types within the testis was possible and this was confirmed by expression of cell specific markers for germ cells (VASA), Sertoli cells (AMH), Leydig cells (3β-HSD) and peritubular myoid cells (SMA).

4.5.2. Differentiation from gonocyte to spermatogonia occurs during fetal and early postnatal life in the human and marmoset

Using a battery of germ cell-specific protein markers the present studies demonstrated sequential germ cell differentiation in the marmoset testis, similar to that described for the human (Hoei-Hansen et al., 2005{Rajpert-De Meyts, 2004 #46);(Honecker et al., 2004);(Gaskell et al., 2004);(Anderson et al., 2007). Expression of proteins required for pluripotency, such as OCT4 and NANOG (Loh et al., 2006), and undifferentiated germ cell (AP-2γ) proteins were demonstrated in the fetal testis of both the human and marmoset. This is consistent with previous reports for the fetal human (Gaskell et al., 2004);(Honecker et al., 2004),(Kerr et al., 2008);(Looijenga et al., 2003a);(Pauls et al., 2006);(Rajpert-De Meyts et al., 2004), with a decreasing proportion of immunopositive germ cells identified as gestation progressed.

Our results, together with previous reports (Rajpert-De Meyts et al., 2004), show that OCT4 is still detected in occasional germ cells in the human at 4-6 months postnatally, whilst NANOG was no longer detected after 4 weeks. In the marmoset the timing of loss of expression of OCT4 occurred between 6 and 14 weeks postnatally, whilst NANOG expression ceased by 6 weeks. This is in keeping with previous suggestions of down-regulation of NANOG occurring earlier in development than OCT4 in human testes (Hoei-Hansen et al., 2005). AP-2γ was expressed until 4-6 months of age in the human testis, in agreement with previous findings (Hoei-Hansen et al., 2004), whilst in the marmoset only occasional germ cells expressed this protein by 6 weeks postnatally.

Previous studies have described gonocytes being present in the marmoset testis well into the juvenile period (Sharpe et al., 2003a);(Chandolia et al., 2006). In these studies cells were characterised based on their position and morphology. The present studies have demonstrated that expression of proteins considered as classical markers of gonocytes are downregulated much earlier in postnatal life than previously suspected. This suggests that the germ cells in previous studies are not in fact gonocytes but are differentiating germ cells that were located in the centre of the tubules rather than adjacent to the basement membrane. In the human it has been reported that spermatogonia do not migrate to the basement membrane until puberty (Yu et al., 2006) and this may also be the case for the marmoset. Identification of central germ cells during the juvenile period as gonocytes is therefore probably misleading.

Proteins associated with differentiating germ cells (VASA, MAGE-A4 and NANOS-1) showed the opposite pattern of expression to those considered characteristic of undifferentiated germ cells in both marmoset and human, consistent with previous studies in the human fetal testis (Anderson et al., 2007);(Aubry et al., 2001). In the human, VASA and MAGE-A4 immunopositive germ cells were detected in second trimester testes, with one report of rare MAGE-A4 positive germ cells in the first trimester (Gaskell et al., 2004). An increasing proportion of the germ cells were immunopositive for these proteins in the third trimester. In the early postnatal period almost every testicular germ cell expressed VASA and MAGE-A4. In the marmoset, MAGE-A4 and VASA were initially detected at 14-15 weeks gestation and the proportion of positive cells increased as gestation progressed. By 6 weeks of postnatal age the majority of germ cells expressed these markers. The increasing proportion of germ cells expressing VASA has also been described in the human (Anderson et al., 2007). Other studies have described the frequency of cells expressing VASA during fetal life as being constant from the second trimester onwards in the human (Honecker et al., 2004), or even reduced from high levels in

the second trimester to a stable level around birth (Cools et al., 2006b). However, these studies involved counting the total number of positive cells rather than the proportion of positive germ cells at each age as in our study.

Based on previous data the OCT4/NANOG/AP-2γ expressing germ cells can be considered to represent gonocytes, whilst the VASA and MAGE-A4 expressing germ cells can be classified as spermatogonia (Gaskell et al., 2004), maturation of the latter involves loss of stem cell/embryonic cell characteristics (Honecker et al., 2004);(Gaskell et al., 2004). The co-stained cells in the marmoset may represent an 'intermediate' population that has been described in the human (Gaskell et al., 2004);(Fukuda et al., 1975).

VASA and NANOS-1 were mostly co-expressed in the same germ cells. Whilst expression of VASA was localised exclusively within the cytoplasm at all ages studied, localisation of NANOS-1 expression changed from the nuclear compartment in fetal life to being present in both nuclear and peri-nuclear cytoplasmic compartments postnatally. This pattern of expression was consistently observed in sections taken from both the human and the marmoset. Peri-nuclear expression of NANOS-1 has been described previously in adult germ cells of the human (Jaruzelska et al., 2003) and mouse (Haraguchi et al., 2003). A similar change in localisation within germ cells has been reported for DAZL, this protein was detected in the nucleus of first trimester germ cells, but was present in both the nucleus and cytoplasm of second trimester germ cells (Anderson et al., 2007). The change in cellular localisation of NANOS-1 suggests that this protein may also have more than one function during germ cell development.

4.5.3. The difference in timing of appearance of the germ cell phenotypes in the human and marmoset reflects the timing of the stages of fetal development

The timing of expression of the germ cell specific proteins during fetal life in the marmoset and human can be considered in relation to the differences in embryology between these two species. Although the pattern of expression is similar in the marmoset, the decrease in expression of gonocyte markers tends to occur comparatively late in gestation compared to the human. VASA immunopositive germ cells were detected from 14/20 weeks' gestation in the marmoset as opposed to 9-14/40 weeks gestation in the human. Similarly the proportion of germ cells expressing OCT4 remained high in the marmoset compared to the human during late gestation. A comparison of the timing of perinatal germ cell differentiation in the marmoset and human testis can be seen in Figure 4.19.

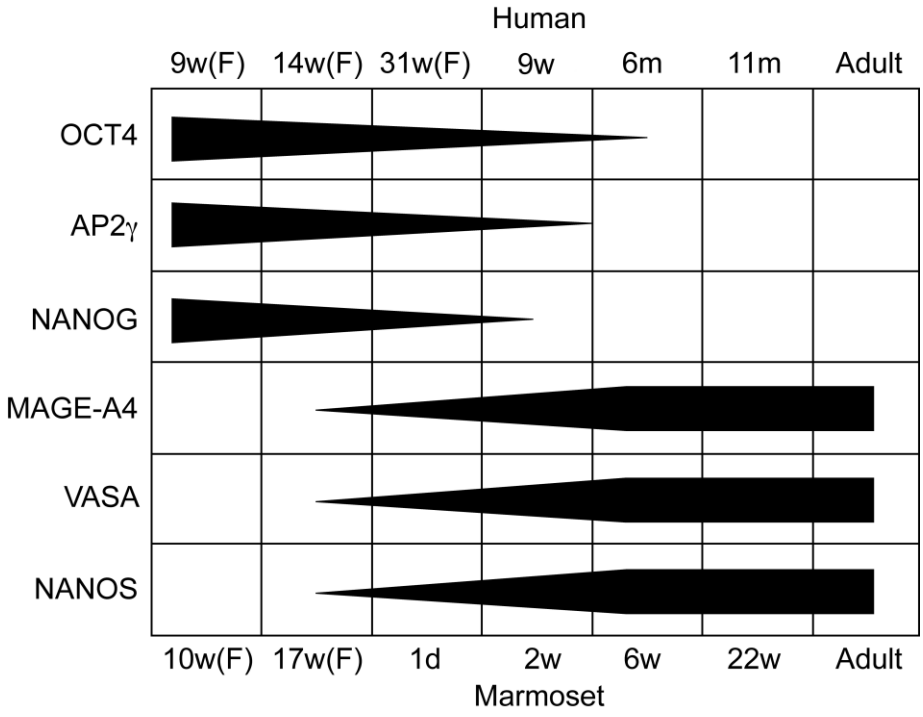

Figure 4.19. Comparison of the timing of expression of germ cell proteins during perinatal life in the human and marmoset. The width of the bars represent proportions of germ cells expressing the protein. F=fetal, w=weeks, m=months.

The reason for the differences in timing of the transition from gonocyte to spermatogonia is likely to be related to the fact that fetal life in the marmoset is characterised by a relatively late implantation and a prolonged embryonic phase such that the fetal phase, during which most of the linear growth occurs, begins at 11/20 weeks gestation (Wistuba et al., 2006), compared to 7/40 weeks in the human (Phillips, 1976). This may explain why proteins that are first expressed in the human during the early part of the second trimester (e.g. VASA) are not expressed in the marmoset until the final third of gestation.

4.5.4. Germ cell differentiation is asynchronous in the marmoset and human, resulting in mixed germ cell populations

The present studies have demonstrated evidence for mixed subpopulations of fetal and early postnatal germ cells within individual seminiferous cords of the marmoset testis. Co-localisation studies revealed a mixed population of germ cells within individual cords, such that a single cord could contain cells expressing an undifferentiated germ cell marker only, a differentiating germ cell marker only or cells in transition that are expressing both markers. Similar subpopulations have also been described in the human for combinations of markers including OCT4/VASA (Anderson et al., 2007);(Honecker et al., 2004), OCT4/MAGE-A4 (Gaskell et al., 2004);(Pauls et al., 2006) and AP-2γ/MAGE-A4 (Pauls et al., 2006) and these subpopulations are associated with morphological changes that occur as gonocytes differentiate into spermatogonia (Fukuda et al., 1975). The intra-cordal mix of germ cells in varying stages of differentiation in the marmoset and human is in marked contrast to the rat, in which all germ cells are synchronised in their differentiation.

We would propose that in the marmoset as in the human the $OCT4^{+}/VASA^{-}$ germ cells are gonocytes, with $OCT4^{+}/VASA^{+}$ being intermediate and $OCT4^{-}/VASA^{+}$ representing spermatogonia (Gaskell et al., 2004). The presence of a mixed

population of germ cells in the human and marmoset testis is in contrast to the rat. In the rat all germ cells appear to be VASA positive on arrival in the developing gonad and there is no evidence of an $OCT4^{+}/VASA^{-}$ population even at e13.5 before the cords have formed (unpublished observation). This has also been demonstrated in the mouse, where VASA is expressed in the germ cells immediately after colonisation of the gonad, but not in migratory primordial germ cells (Toyooka et al., 2000). In the rat, by e19.5 all of the germ cells in every cord were OCT4 negative but continued to express VASA. Indeed, in most germ cells, cessation of OCT4 expression is complete by as early as e17.5 (Ferrara et al., 2006). The transition from gonocyte to spermatogonia occurs over a very short time period in the rat, when compared to the human and marmoset in which it takes many months for all the cells to differentiate. In the human and marmoset testis this process spans much of fetal life and also extends into early postnatal life, as opposed to the rat in which the process occurs over a period of 2 days and is confined to a short period of fetal life.

4.5.5. Germ cell proliferation is asynchronous in the marmoset and human, resulting in mixed germ cell populations

In addition to germ cell differentiation, the present studies have also demonstrated for the first time that proliferation of germ cells occurs in a variable proportion of germ cells in the marmoset during all fetal ages investigated from 11 weeks' gestation until early postnatal life and that this proliferation occurs in a proportion of both undifferentiated (AP-2γ positive gonocytes) and differentiated (VASA positive spermatogonia) germ cells during this period. Proliferation of germ cells occurs in the human during the first and second trimester and has been reported to continue in the third trimester and into early postnatal life (Honecker et al., 2004), similar to the proportions seen in the fetal marmoset. In contrast, the rat exhibits a synchronised pattern of germ cell proliferation in which a high proliferation index occurs at e15.5 followed by a complete cessation of proliferation for the remainder of gestation until postnatal days 4-6 (Ferrara et al., 2006), and a similar period of

quiescence also occurs in the mouse (Vergouwen et al., 1991);(Culty, 2009). This proliferative quiescence includes the period of differentiation of germ cells from gonocyte to spermatogonia. A period when testicular germ cell proliferation has ceased has not been found in the marmoset or human and proliferation continues to occur during the transition of germ cells from gonocyte to spermatogonia.

4.5.6. Asynchronous development of germ cells may predispose to CIS and TGCT

The apparently gradual and asynchronous differentiation and proliferation of fetal germ cells in the marmoset and human is intriguing and may have implications regarding the development of CIS and TGCT. It is well recognised that CIS cells (the precursor lesion for TGCT), closely resemble human fetal germ cells in terms of both morphology (Gondos, 1993) and protein expression, including NANOG, OCT4, AP-2γ, VASA and MAGE-A4 (Rajpert-De Meyts, 2006). These expression patterns support the hypothesis that CIS cells arise from immature fetal germ cells that fail to differentiate completely into normal spermatogonia (Skakkebaek et al., 1987). Germ cell differentiation occurs over a prolonged period in humans and this may, given the right circumstances, predispose to the development of CIS (Rajpert-De Meyts, 2006). Indeed in humans with some disorders of sex development there is evidence of delayed germ cell differentiation, based on persistence of OCT4 expression. These patients have an increased risk of CIS and TGCT in later life (Cools et al., 2006a). The presence of several subpopulations of germ cells at different stages of differentiation and proliferation, all exposed to the same local environment within the seminiferous cords during fetal or early postnatal life may also increase the likelihood of incomplete differentiation of some of the more immature germ cells and thus increase the risk of developing CIS.

In humans the development of CIS and TGCT has been proposed to be part of a testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2007). A combination of

features similar to human TDS can be induced in the rat using phthalates (Fisher et al., 2003), but this does not include the development of CIS or TGCT. This failure to induce CIS in the rodent may be related to the way in which the fetal germ cells differentiate. If asynchronous and prolonged differentiation, with several subpopulations of germ cells co-existing in different stages of differentiation, predisposes to malignant transformation in the human (Rajpert-De Meyts, 2006), then CIS may also occur spontaneously or be inducible in the marmoset. However to date there are no published reports of testicular tumours in this species, which could suggest that other predisposing factors may be required. These factors may include exposure to environmental factors such as phthalates.

4.5.7. Postnatal germ cell differentiation and proliferation in the marmoset has similarities to the human

The postnatal marmoset testis continues to express proteins such as VASA, MAGE-A4 and NANOS-1. The cell types expressing these proteins are identical to those described in the human. Expression of VASA is weak in spermatogonia, with strong staining in spermatocytes and spermatids in the human adult testis (Castrillon et al., 2000), which exactly matches the expression seen in the marmoset. Equally, MAGE-A4 staining has been described to be strong in spermatogonia with faint staining in primary spermatocytes and no expression in later spermatocytes and spermatids (Aubry et al., 2001), as has been described in the present study for the marmoset testis. Importantly, in the context of CIS and TGCT, proteins associated with fetal germ cells and CIS (e.g. OCT4, NANOG, PLAP) in the human are not identified in germ cells in either normal adult human (Looijenga et al., 2007a) or marmoset testis. This means that identification of OCT4 expression beyond the infancy period in either human or marmoset testes would indicate delayed differentiation of gonocytes or development of CIS (Oosterhuis and Looijenga, 2005);(Cools et al., 2006b). The cessation of expression of OCT4 that occurs on differentiation from gonocyte to spermatogonia in the human and marmoset contrasts with the situation

in the prepubertal and adult mouse in which stem cell spermatogonia retain expression of Oct4 (Pesce et al., 1998);(Ohbo et al., 2003);(Tadokoro et al., 2002). KIT expression is bimodal in germ cells of the marmoset testis. In fetal life KIT is expressed in the gonocytes (Robinson et al., 2001) and these cells are distinct from the MAGE-A4 expressing differentiating germ cells as has been described in the human (Gaskell et al., 2004). As the gonocytes differentiate in fetal and early postnatal life the proportion of KIT expressing germ cells decreases, however KIT begins to be expressed in the differentiating spermatogonia of the juvenile and adult testis. Unlike the gonocyte population these KIT expressing cells co-express MAGE-A4. This bimodal expression of KIT also occurs in the human (Unni et al., 2009);(Gaskell et al., 2004) and the mouse (von Schonfeldt et al., 2004);(Culty, 2009);(Prabhu et al., 2006). The interpretation of results of KIT staining must therefore be considered in the context of the age and stage of development of the testis in these species.

Germ cell proliferation continues in the marmoset from fetal into postnatal life. This has also been demonstrated during early postnatal development in the human fetal testis (Honecker et al., 2004), although another study did not detect proliferating germ cells during the neonatal period (Ketola et al., 2003). A relatively high germ cell proliferation index was found in the neonatal period in the marmoset, followed by a reduced index during the childhood phase. With the onset of puberty the proliferation index increased to levels comparable to those during the neonatal period. A relatively high proliferation index has also been shown to occur in the spermatogonia of the adult human testis, with 26% of spermatogonia expressing Ki67 (Steger et al., 1998). The pattern of germ cell proliferation in the marmoset corresponds to the level of gonadotrophins and testosterone during the three phases of development, although a direct association between germ cell proliferation and levels of these hormones has not been demonstrated. This finding led to investigation of experimental suppression the gonadotrophins and testosterone and

its effect on proliferation during the childhood phase in the marmoset (Kelnar et al., 2002). Suppression of gonadotrophins and testosterone did not affect germ cell proliferation during this period, however the effect on germ cell proliferation or differentiation has not been investigated during the neonatal period when levels of both are high. Such an effect could be relevant to disorders that involve delayed or abnormal development of gonocytes, such as CIS and TGCT (Cools et al., 2005). The effects of treatment with GNRHa on neonatal germ cell development will be investigated in chapter 6.

4.5.8. Conclusion

This chapter has demonstrated that germ cell differentiation and proliferation in the marmoset is comparable to that of the human during fetal and postnatal life. This makes the marmoset suitable for studies that may have direct relevance to human testis development. During fetal life, gonocytes differentiate into spermatogonia. This process occurs over a relatively prolonged period of time and is asynchronous in the marmoset and human. This results in the presence of germ cells at several stages of differentiation within individual seminiferous cords. This is in marked contrast to the rodent in which more synchronous germ cell differentiation and proliferation occurs during fetal and early postnatal life, during a relatively narrow time window. This pattern of germ cell differentiation may explain why rodents do not develop CIS and TGCT and this would imply that the marmoset could be predisposed to develop these tumours, although no such tumours have been previously described. Having established the marmoset as a comparable model of germ cell development to the human the next chapter will investigate the development of an *in vivo* xenografting model to recapitulate germ cell development in perinatal marmoset and fetal human testes.

5 Testicular xenografting as an *in vivo* model of testis development in the human and marmoset

5.1. Introduction

5.1.1. Approaches for manipulation of testis development in the human and marmoset

The previous chapter demonstrated that the marmoset is a relevant model for germ cell differentiation and proliferation in the human testis. Further study of testis development, including perturbation of normal development could potentially be achieved using one of three possible approaches. The first approach would be to administer interventions to the animal directly. Alternatively, tissue could be removed from the animal and treated under *in vitro* conditions. Both of these approaches have limitations as some treatments might not be acceptable and the *in vitro* systems may not recapitulate normal development. Therefore an *in vivo* system that can be achieved ex-situ could be useful for such studies. One such approach is to xenograft testis tissue fragments into a rodent host animal and study the functional development of the grafts. Treatments can then be administered to the host animal to affect development of the grafts.

5.1.2. Xenografting as an *in vivo* system of testis development

Testis xenografting has been shown to sustain tissue survival and promote differentiation of germ cells in several species (Honaramooz et al., 2002). In rodents, testis tissue taken from immature animals and grafted into immunodeficient hosts exhibits germ cell progression through meiosis and can produce functional spermatozoa, capable of producing progeny following intracytoplasmic sperm injection (Schlatt et al., 2003). Potential applications of this technique include fertility preservation and restoration, preservation of endangered species and production of transgenics (Griswold et al., 2001), however the fact that this technique can sustain

normal testicular architecture and recapitulate normal development of germ cells may also make it useful in studies of early germ development and perturbation in human and non human primates.

5.1.3. Conditions required for testis xenografting

The hormonal environment is important for the normal development of the testis and the same is true for xenografts. It is generally recommended for the host animal to be castrated (Rodriguez Sosa and Dobrinski, 2009), in order to reduce negative feedback by androgen on the pituitary and thereby stimulate the release of gonadotrophins, which in turn can induce testosterone production from the grafts. High intratesticular concentrations of testosterone are essential for normal testis development and studies in rhesus monkey grafts have reported that castration is essential for development of the grafts (Honaramooz et al., 2004). Most studies have described introducing grafts of 0.5-1mm^3 although evidence for the optimum size of grafts is lacking (Rodriguez Sosa and Dobrinski, 2009). Temperature is also important for the success of xenografting and therefore the choice of site for grafts may be important. Most studies insert grafts under the back skin of a nude mouse and the outcome is satisfactory, however hyperthermia has been cited as a possible cause of less successful outcomes in some subcutaneous grafts such as those in primates (Wistuba et al., 2004).

5.1.4. Xenografting of human and marmoset testis tissue

Xenografting of human and non-human primate testis material has been performed for postnatal testes. In the rhesus monkey this can result in the completion of spermatogenesis (Honaramooz et al., 2004). However as discussed previously it is known that the rhesus monkey exhibits an organisation of spermatogenesis that shows closer resemblance to the rodent than the human (Millar et al., 2000). To date, xenografting of postnatal human and marmoset testes have not resulted in progression through meiosis (Schlatt et al., 2002);(Goossens et al., 2008).

5.1.5. Xenografting of fetal testis tissue

There are few studies on fetal testis development using this approach (Yu et al., 2006);(Skakkebaek et al., 1974) and it remains to be established whether this system is able to recapitulate normal fetal germ cell development in either the human or non human primate testis.

5.2. Chapter aims

This chapter aimed to investigate the potential of a testis xenografting approach to recapitulate normal development in the human and marmoset by grafting fetal and postnatal testis tissue and comparing the development with non grafted controls of comparable ages.

5.3. Materials and methods

5.3.1. Neonatal mouse testis tissue collection

Neonatal mouse testes (n=7) were collected as described in section 2.4.4.

5.3.2. Human fetal testis tissue collection

Human fetal testis tissue for use in immunohistochmical studies was obtained as described in Section 2.4.2. The sections used were first trimester fetuses (9 weeks gestation, n=4), second trimester fetuses (14, 15, 16, 17, 18 weeks gestation; n=1 for each age).

5.3.3. Fetal and postnatal marmoset testis collection

Marmoset testes from fetal (15 weeks, n=1; 16 weeks, n=1; 18 weeks, n=1; and postnatal (1 day, n=2; 25 weeks, n=1) animals were obtained as described in section 2.4.1.

5.3.4. Xenografting

Castration and xenografting of testis tissue was performed as detailed in section 2.6.

5.3.5. SRY genotyping

The sex of first trimester human fetuses was established by SRY genotyping as described in section 2.6.4.

5.3.6. Haematoxylin and eosin staining

The method for haematoxylin and eosin staining is described in section 2.9.

5.3.7. Immunohistochemistry

Single immunohistochemistry was performed with DAB detection as described in section 2.10. The primary antibodies used for these experiments are listed in Table 2.1. Double staining was performed with DAB and fast blue detection as outlined in section 2.10.3. Details of conditions for double staining experiments can be found in Table 2.2.

5.3.8. Immunofluorescence

Triple immunofluorescence was performed as described in section 2.11.2. The primary and secondary antibodies, and detection labels used for these experiments are listed in Table 2.4.

5.3.9. Serum testosterone radioimmunoassay

Serum testosterone levels were measured in host mice as described in section 2.7.

5.3.10. Calculation of the germ cell to Sertoli cell ratio and quantification of germ cell subpopulations in human fetal testis xenografts

Sections of pre-graft controls, xenografts and post-graft controls were analysed. For the first trimester xenografts, pre-graft controls were obtained using tissue from the same testis prior to grafting. Second trimester control tissue acted as the post-graft

control for first trimester xenografts, as well as a pre-graft control for the second trimester xenografts. Post-graft controls for second trimester xenografts were obtained using tissue taken from 20 week gestation fetuses.

The ratio of Sertoli cells per germ cell was calculated following double staining for OCT4 and VASA, which together stained all fetal germ cells. The total number of germ cells was calculated by adding together the unstained (Sertoli) cells and dividing them by the total number of stained (germ) cells. Quantification of subpopulations of germ cells in double stained cells is described in section 2.12.1, The proportion of germ cells expressing the various combinations of markers were calculated by dividing the number of germ cells with a particular expression profile by the total cells, for a minimum of 40 random fields. Statistical analysis was performed using a one-way analysis of variance (ANOVA). Triple stained immunofluorescent sections were analysed as described in section 2.12.2.2. Quantification of sections from pre-graft controls, xenografts and post-graft controls was also undertaken. The proportion of germ cells expressing the different combinations of markers was calculated as described above and a proliferation index for each subpopulation was calculated by dividing the number of proliferating (Ki67 positive) cells by the total number of cells within that subpopulation. Statistical analysis was performed using the unpaired students t-test for two groups or one-way ANOVA for multiple groups.

5.4. Results

5.4.1. Neonatal mouse testis allografting

In order to complement the *in vivo* work in the perinatal marmoset, we wished to use a xenografting approach for investigating germ cell development during this period. Firstly we needed to establish the system within our institution and to do this we used neonatal mouse testes as the donor tissue in order to reproduce results

obtained in other laboratories. Testis pieces were grafted subcutaneously for 2, 4 or 8 weeks and the grafts showed excellent survival, comparable to recent reports (Yu et al.) and substantially better than early reports (Schlatt 2003), (Honaramooz 2002) (Table 5.1).

Grafting duration (weeks)	Grafts (total number)	Grafts retrieved (%)	Grafts with testis cords (%)
2	12	92	67
4	18	100	72
8	18	100	72

Table 5.1. Success rate for neonatal mouse testis allografts.

Castration of the host meant that serum testosterone measurement in the host animal reflected testosterone production by the grafts. In addition, seminal vesicle weight of the host could also be used as a surrogate marker for testosterone production. In castrate animals with no recoverable grafts the serum testosterone was below the limit of detection (<0.1ng/ml) and mean seminal vesicles weight was 31.5mg, whilst intact host animals had mean serum testosterone levels of 6.1 ng/ml and seminal vesicle weight of 264mg. In castrate animals with grafts the serum testosterone and seminal vesicle weights indicated that the grafts were producing testosterone (Table 5.2).

Control/Grafted (duration)	Castrate/Intact	Testosterone		Seminal vesicle	
		ng/ml	range	mg	range
Control	Castrate	<0.1	(all <0.1)	31.5	(23-40)
Grafted (4w)	Castrate	1.0	(0.6–1.3)	103	(69-120)
Grafted (8w)	Castrate	2.7	(1.8-3.7)	213	(160-151)
Control	Intact	6.1	(0.3-11.8)	264	(209-318)

Table 5.2. Serum testosterone and seminal vesicle weight for hosts receiving neonatal mouse testis allografts. Control – animals with no recoverable testis in grafts. w=weeks.

The morphology of the seminiferous tubules in grafts was similar to controls of equivalent age at each time point analysed. In addition grafts that had been left in-situ for 8 weeks had undergone complete spermatogenesis with the production of elongate spermatids and this was associated with a higher testosterone and seminal vesicle weight, compared with the 4 week grafts (Figure 5.1). These results validated the grafting technique for use in subsequent xenografting experiments.

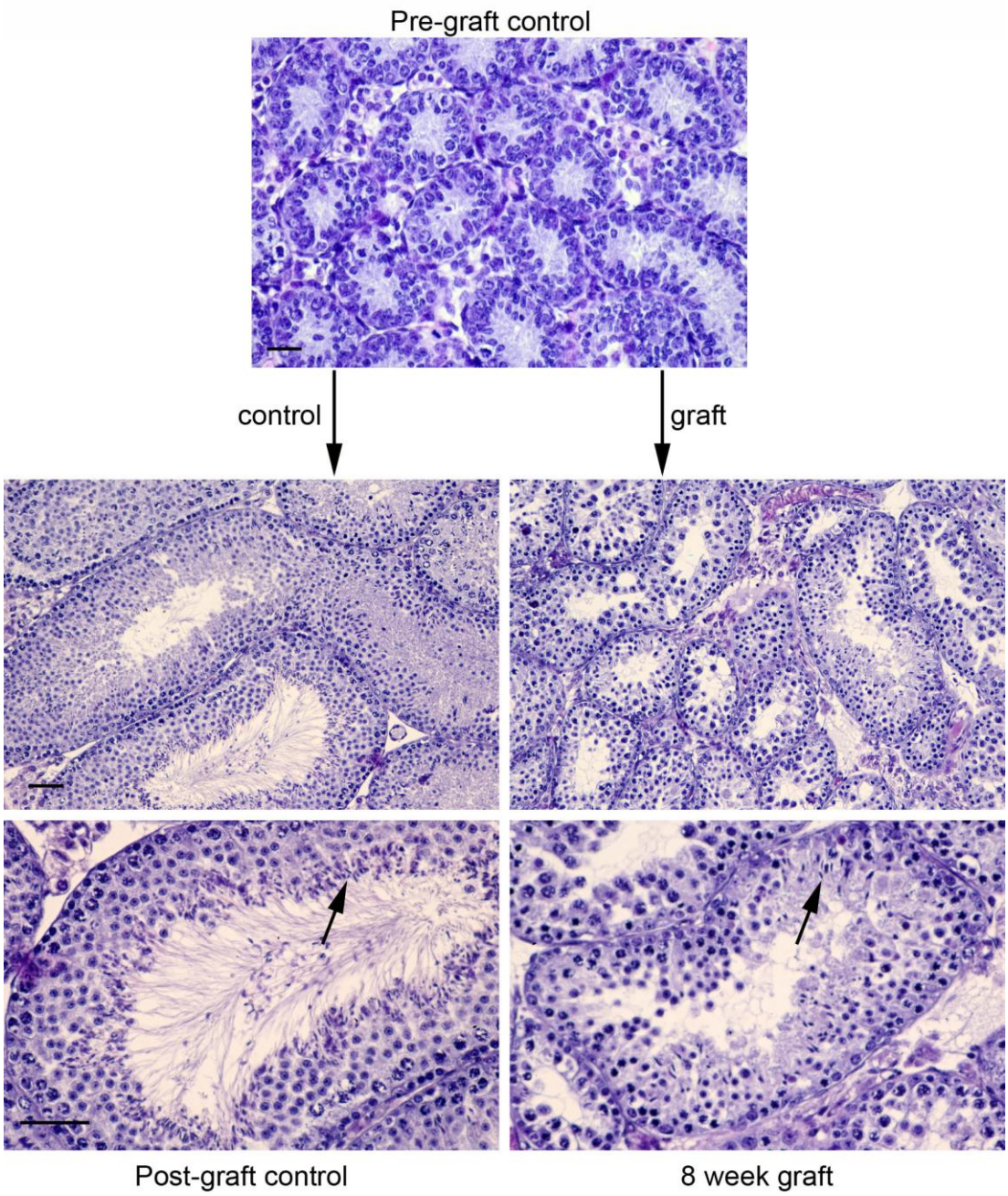

Figure 5.1. H+E. Allografting of neonatal mouse testis. Allografts retrieved after 8 weeks (right panels) compared with testis from an 8 week old control animal (left panels). Note that both control and grafted tissue exhibit complete spermatogenesis with appearance of elongate spermatids (arrows). Scale bar = 50μm. Original magnification 40x (middle panels x20).

5.4.2. Marmoset testis xenografts

5.4.2.1. Marmoset fetal testis xenografts

Having established the grafting technique we then wished to investigate the suitability of this approach for studying early germ cell development in the fetal marmoset, as this species has been shown to represent a good model for human germ cell development (Chapter 4). Testes from 15, 16 and 18 week gestation marmosets were grafted for 6 weeks and a high proportion of these grafts were retrieved, with easily identifiable testis cords in most of these grafts. In addition there had been a significant growth of the grafts from the original 1mm in diameter up to 3mm post grafting (Figure 5.2).

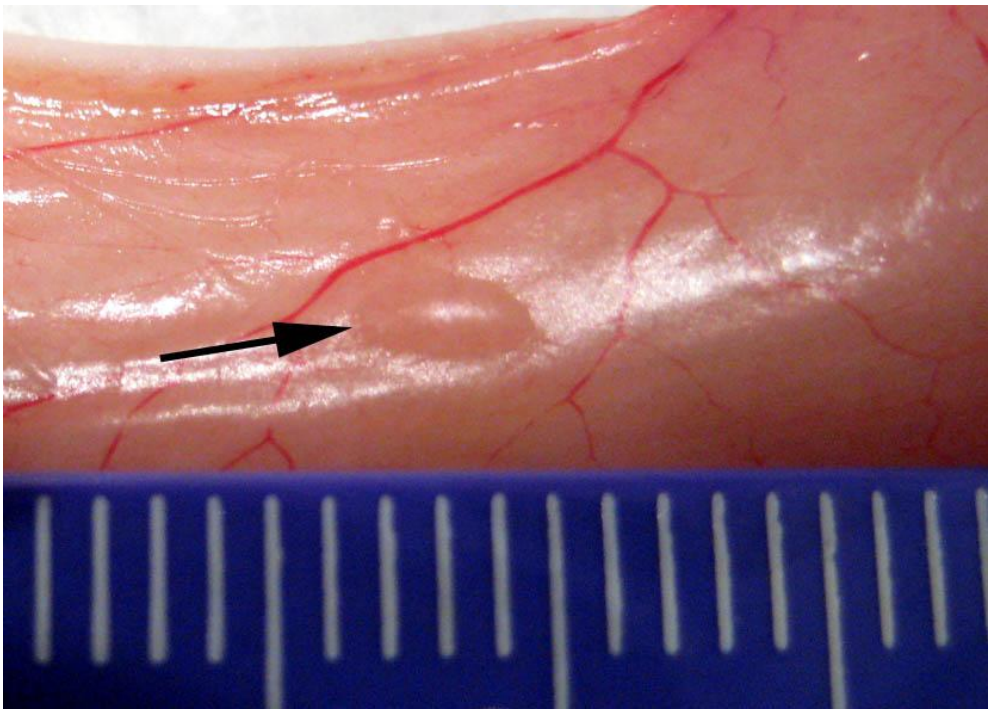

Figure 5.2. Subcutaneous testis xenograft from an 18 week fetal marmoset donor retrieved after 6 weeks. Note that the diameter of the graft has increased to approximately 3mm.

The success rate was lower in the grafts from 15-16 week fetuses, with fewer cords observed in these grafts, compared to the grafts from 18w fetal marmoset grafts (Table 5.3).

Age (weeks)	Grafting duration (weeks)	Grafts (total number)	Grafts retrieved (%)	Grafts with testis cords (%)
15	6	12	100	75
16	6	12	67	58
18	6	9	100	100

Table 5.3. Success rate for fetal marmoset testis xenografts.

Serum testosterone and seminal vesicle weights indicated that the grafts were producing small amounts of testosterone. The testosterone production was much reduced compared to ungrafted non-castrate control mice at the equivalent age (Table 5.4).

Control/Grafted (duration)	Castrate/Intact	Testosterone		Seminal vesicle	
		ng/ml	range	mg	range
Control	Castrate	<0.1	(all <0.1)	31.5	(23-40)
Grafted (6w)	Castrate	0.11	(0.1–0.17)	49	(37-66)
Grafted (6w)	Intact	0.99	n/a	249	n/a
Control	Intact	6.1	(0.3-11.8)	264	(209-318)

Table 5.4. Serum testosterone and seminal vesicle weight for hosts receiving fetal marmoset testis xenografts. Control – animals with no recoverable testis in grafts. w=weeks.

Grafts from 15 week fetal marmoset testes retained seminiferous cord structure and the cords contained large numbers of germ and Sertoli cells (Figure 5.3).

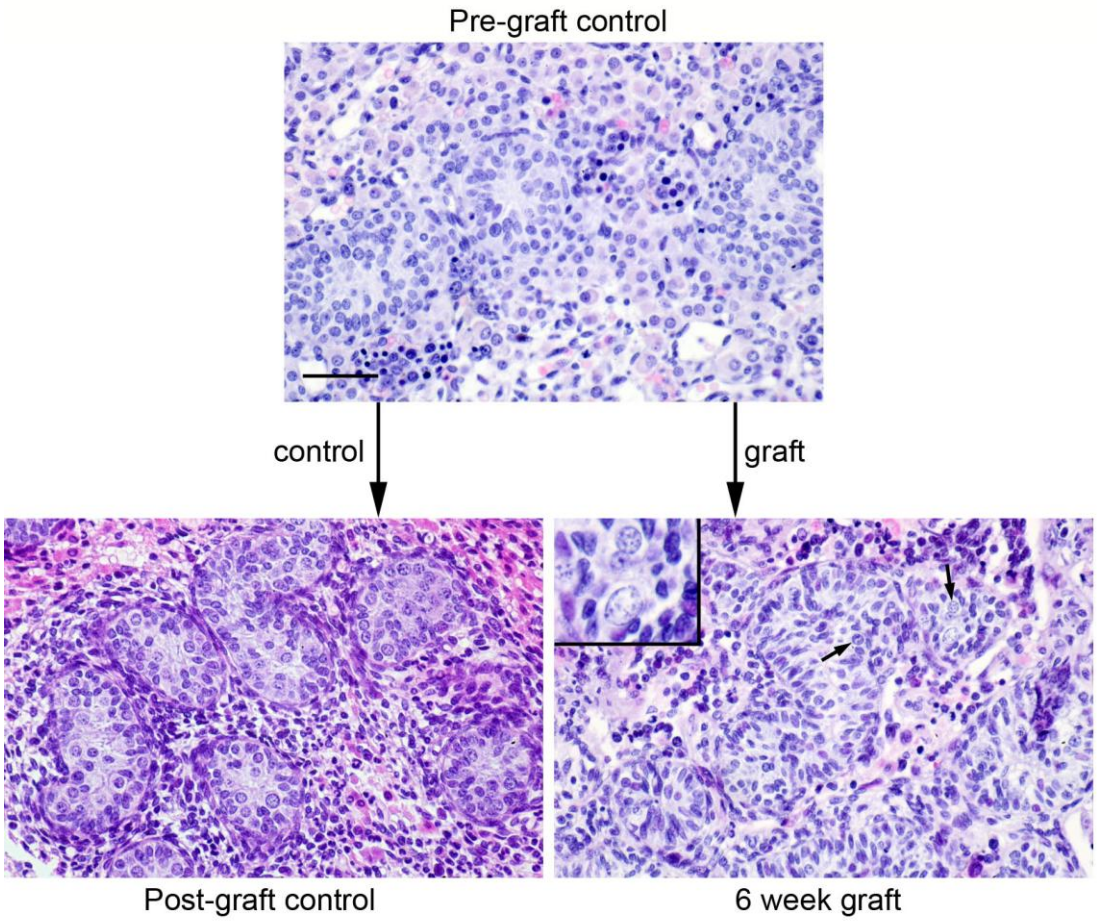

Figure 5.3. H+E. 15 week fetal marmoset testis xenografts. Xenograft of a 15 week fetal marmoset testis retrieved after 6 weeks (right panel) and comparison with testis from a neonatal control animal (left panel). Germ cells (right panel, inset) are present within the graft (arrows). Scale bar = 50μm.

Grafts from 18 week gestation testes showed excellent morphology (Figure 5.4a). The grafts had formed an oval capsule structure giving them a distinct border and making them easy to dissect from the under surface of the skin (Figure 5.4b). These fetal grafts expressed markers of, Sertoli (AMH, cytokeratin), Leydig (3β-HSD) and peritubular myoid (AR) cells (Figure 5.5).

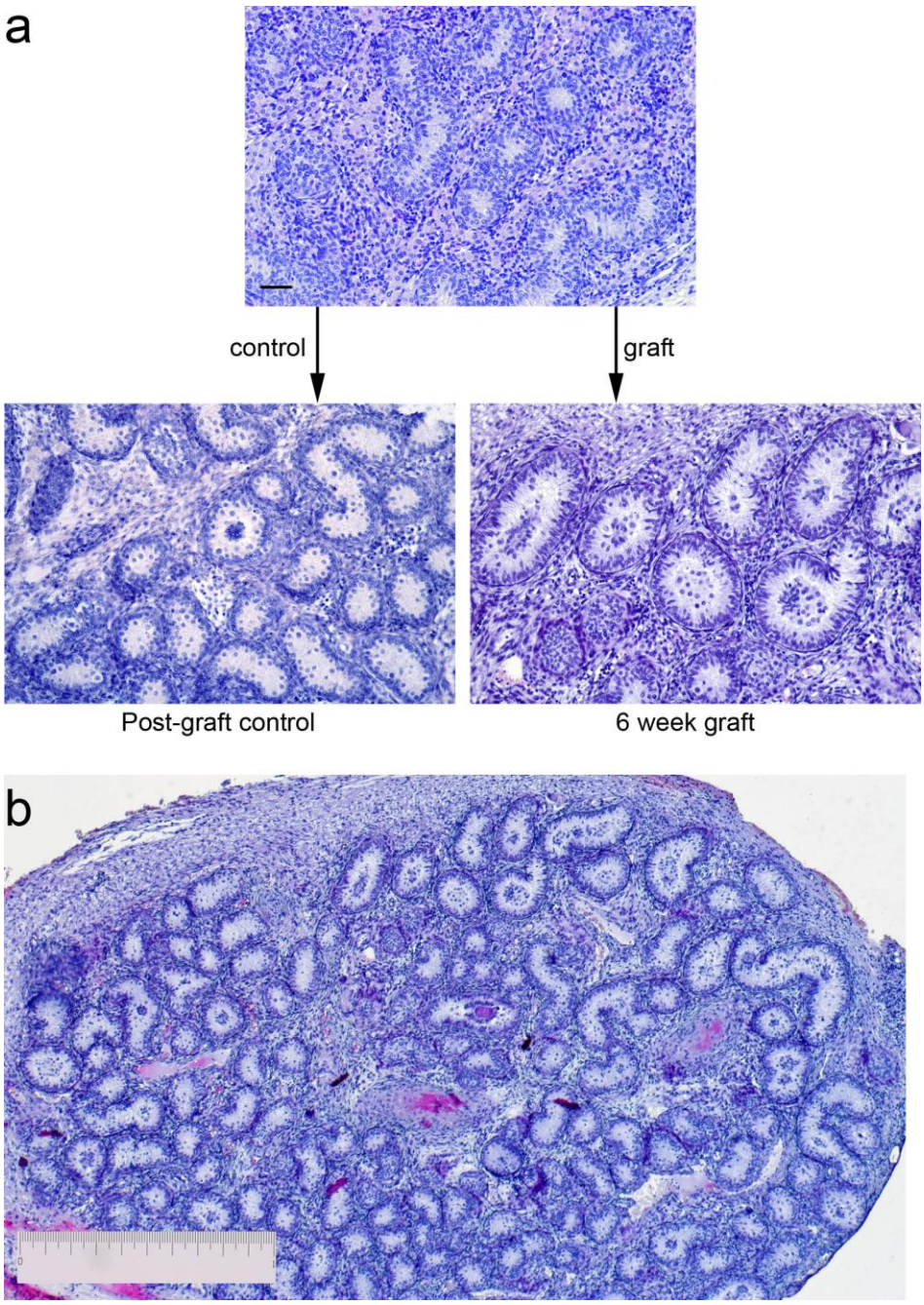

Figure 5.4. H+E. 18 week fetal marmoset testis xenograft. a) H+E. Xenograft of an 18 week marmoset testis retrieved after 6 weeks (right panel) and comparison with testis from a 6 week postnatal control animal (left panel). Scale bar = 50μm. b) H+E. Low power image (x4) of graft in a). Note that the cords are distributed throughout the graft and the graft has taken up the ovoid shape of a testis with a surrounding 'capsule'. Scale bar = 1mm.

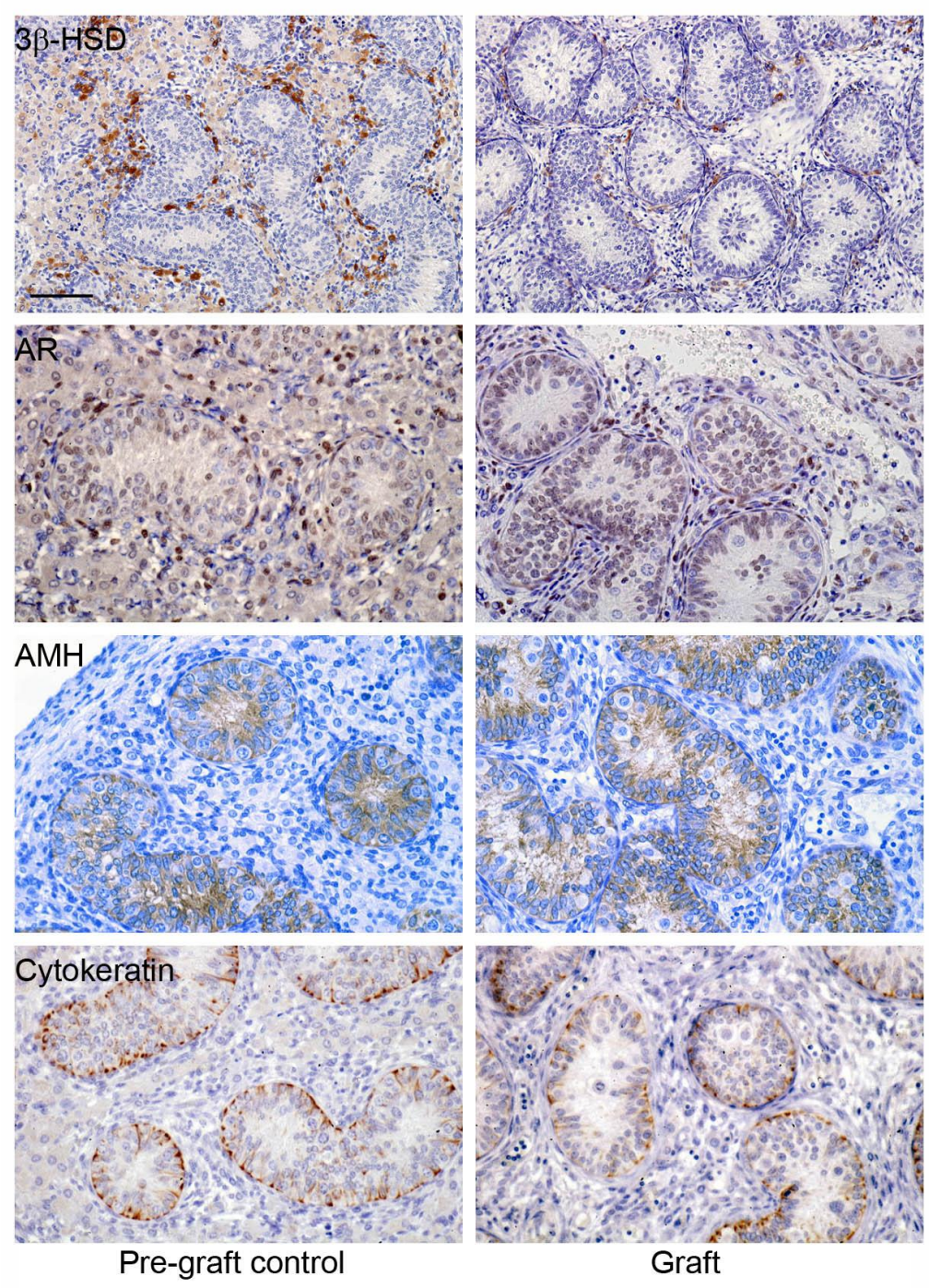

Figure 5.5. Expression of 3β-HSD, AR, AMH and pan-cytokeratin in 18w fetal marmoset xenografts. Grafts were retrieved after 6 weeks (right panels), and compared with testis from the pre-graft tissue (left panels). Note that both control and grafted tissue has a full complement of testicular somatic cell types. Scale bar = 50μm.

The integrity of the basement membrane in grafts from fetal marmosets is demonstrated by the strong expression of SMA at the periphery of the cords. In addition the structure of the vasculature and the integrity of the endothelial cells can also be observed using this marker (Figure 5.6).

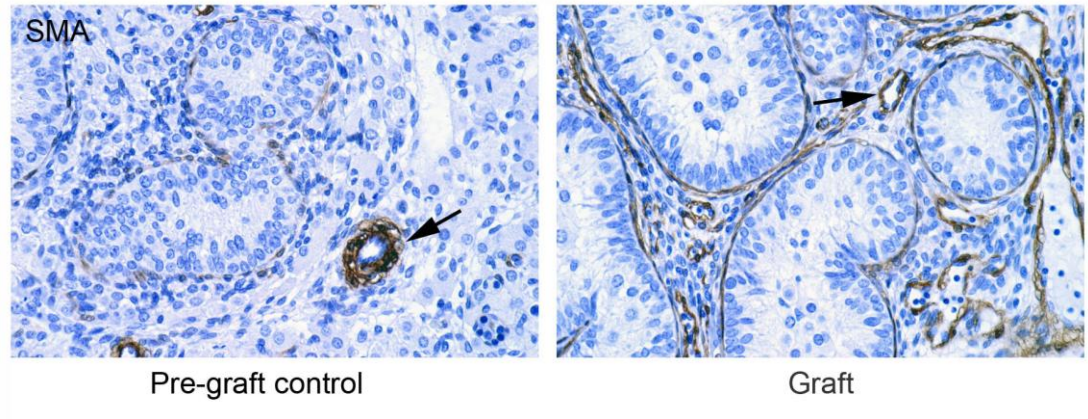

Figure 5.6. SMA expression in an 18w fetal marmoset testis xenograft. Grafts were retrieved after 6 weeks (right panel), and compared with the pre-graft tissue (left panel). Expression of SMA in vascular smooth muscle cells is seen in both control and graft (arrows). Scale bar = 50μm.

Germ cell differentiation occurred in grafts from fetal marmosets (Figure 5.7). The expression of OCT4 and AP-2γ decreased after grafting, whilst the expression of VASA and MAGE-A4 increases (Figure 5.7). This progression is comparable to the situation in the equivalent age post graft control, suggesting that the grafting technique mimics germ cell differentiation in the in-situ marmoset testis.

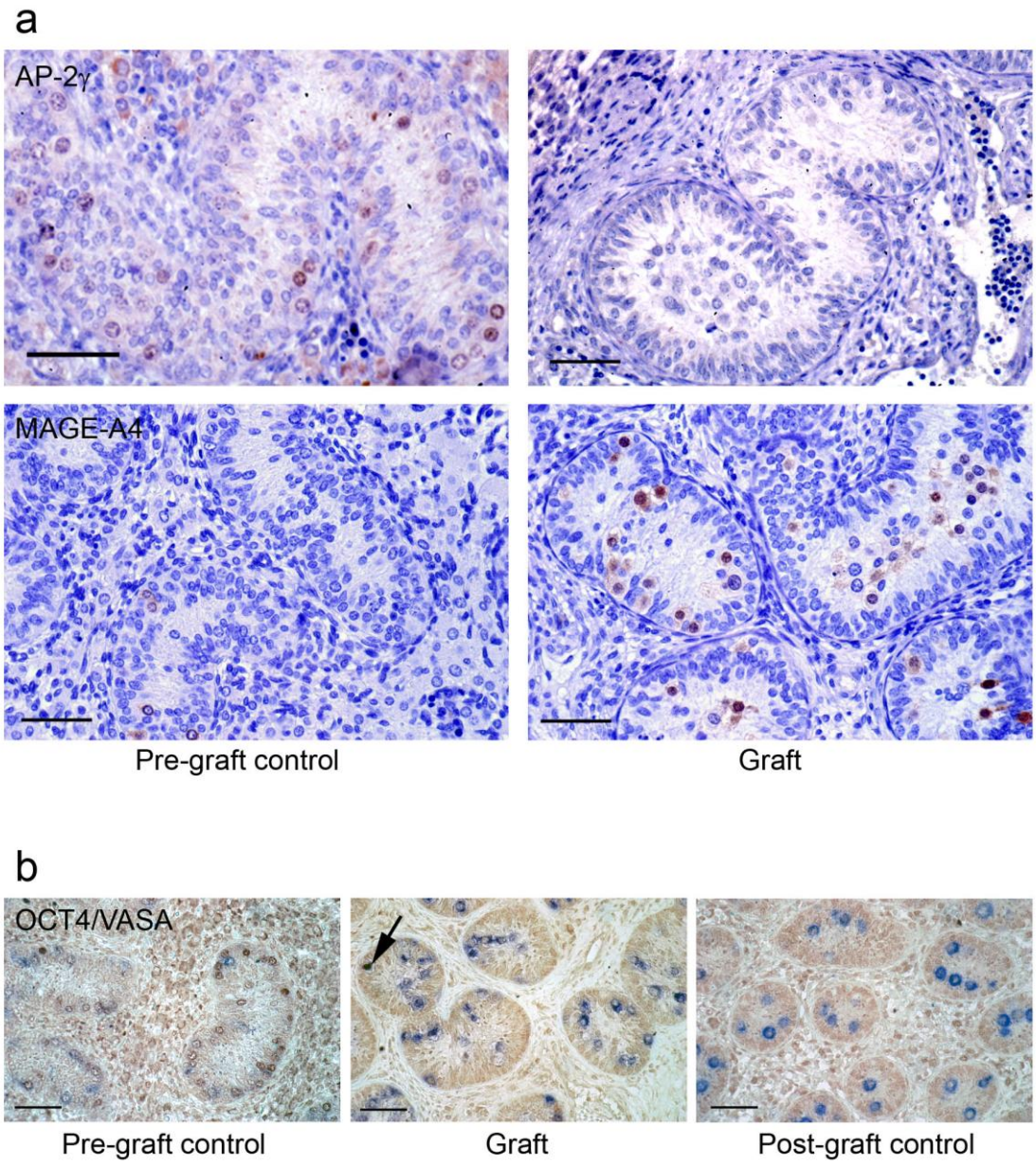

Figure 5.7. Expression of AP-2γ, MAGE-A4 and OCT4/VASA in a xenograft of an 18 week fetal marmoset testis. Grafts were retrieved after 6 weeks. a) AP-2γ expression decreases, whilst MAGE-A4 expression increases after grafting. b) OCT4 (brown) is also reduced during grafting (arrows), whilst VASA (blue) expression increases. Scale bar = 50μm. Original magnification (a) 40x, (b) 20x.

5.4.2.2. Neonatal marmoset testis xenografts

Testes from neonatal marmosets were grafted for 2 or 6 weeks and demonstrated excellent survival with all of the grafts retrieved and most of the grafts exhibiting testis cords (Table 5.5).

Grafting duration (weeks)	Grafts (total number)	Grafts retrieved (%)	Grafts with testis cords (%)
6	14	100	79

Table 5.5. Success rate for neonatal marmoset testis xenografts.

Serum testosterone and seminal vesicle weights indicated that these grafts were not producing testosterone. Serum levels of testosterone were below the limit of detection and mean seminal vesicle weight was 22mg (19-28). Morphology of the tubules was variable with occasional grafts exhibiting marked degeneration of the tubules, however most of the grafts demonstrated morphology characteristic of ungrafted control testis of the same age with all testis cell types identified (Figure 5.8).

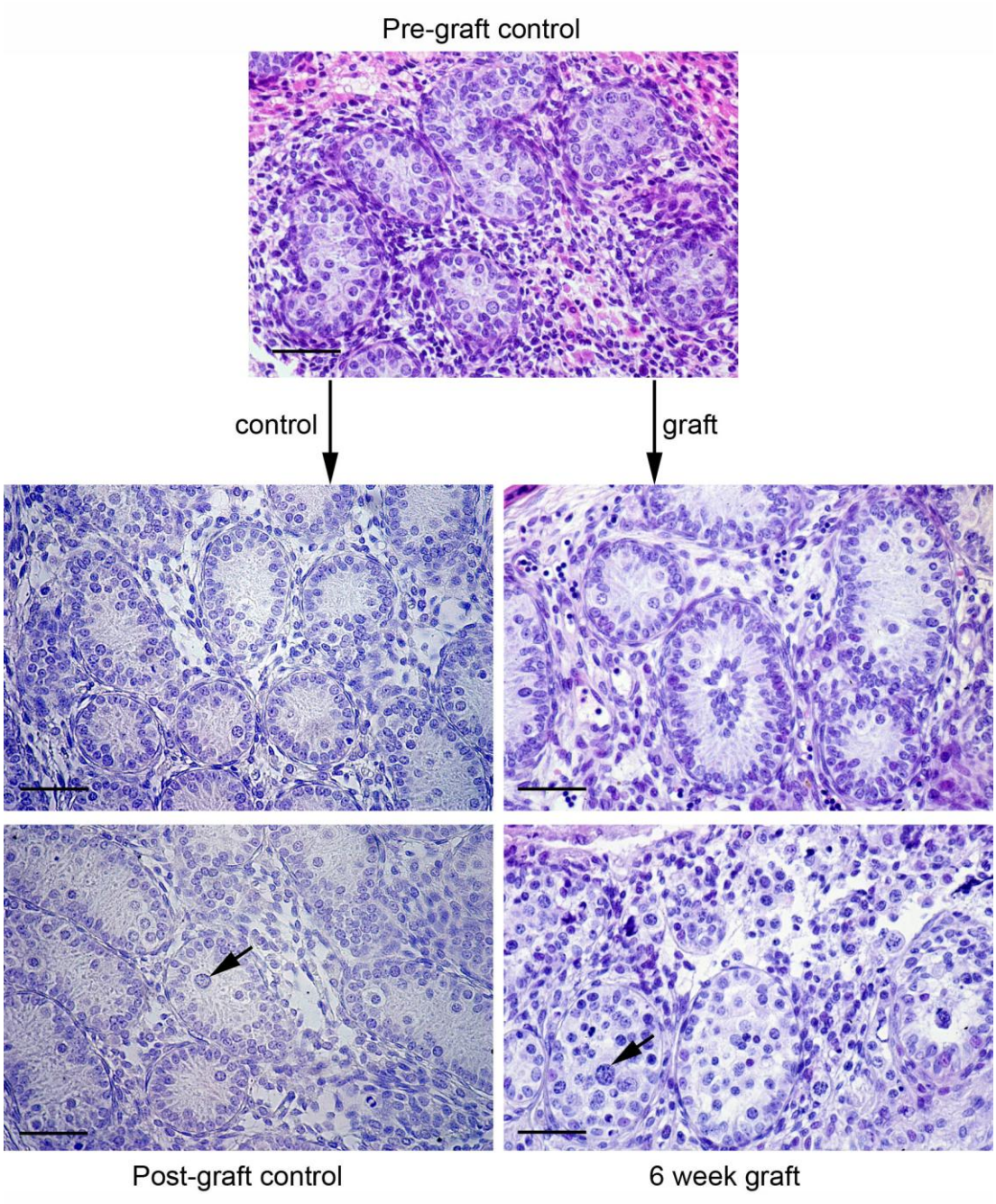

Figure 5.8. H+E. Xenograft of neonatal marmoset testis. Grafts were retrieved after 6 weeks (right panels) and compared with testis from a 6 week postnatal control animal (left panels). Note that both control and grafted tissue has a full complement of cell types including germ cells (arrows). Scale bar = 50μm.

Grafts were compared to pre graft testes in addition to those taken from non grafted animals at 6 weeks of postnatal age (post graft controls). Expression of 3β-HSD, androgen receptor, AMH and pan cytokeratin were demonstrated in all three conditions, which showed the presence of Leydig, Sertoli and peritubular myoid cells within the grafts (Figure 5.9).

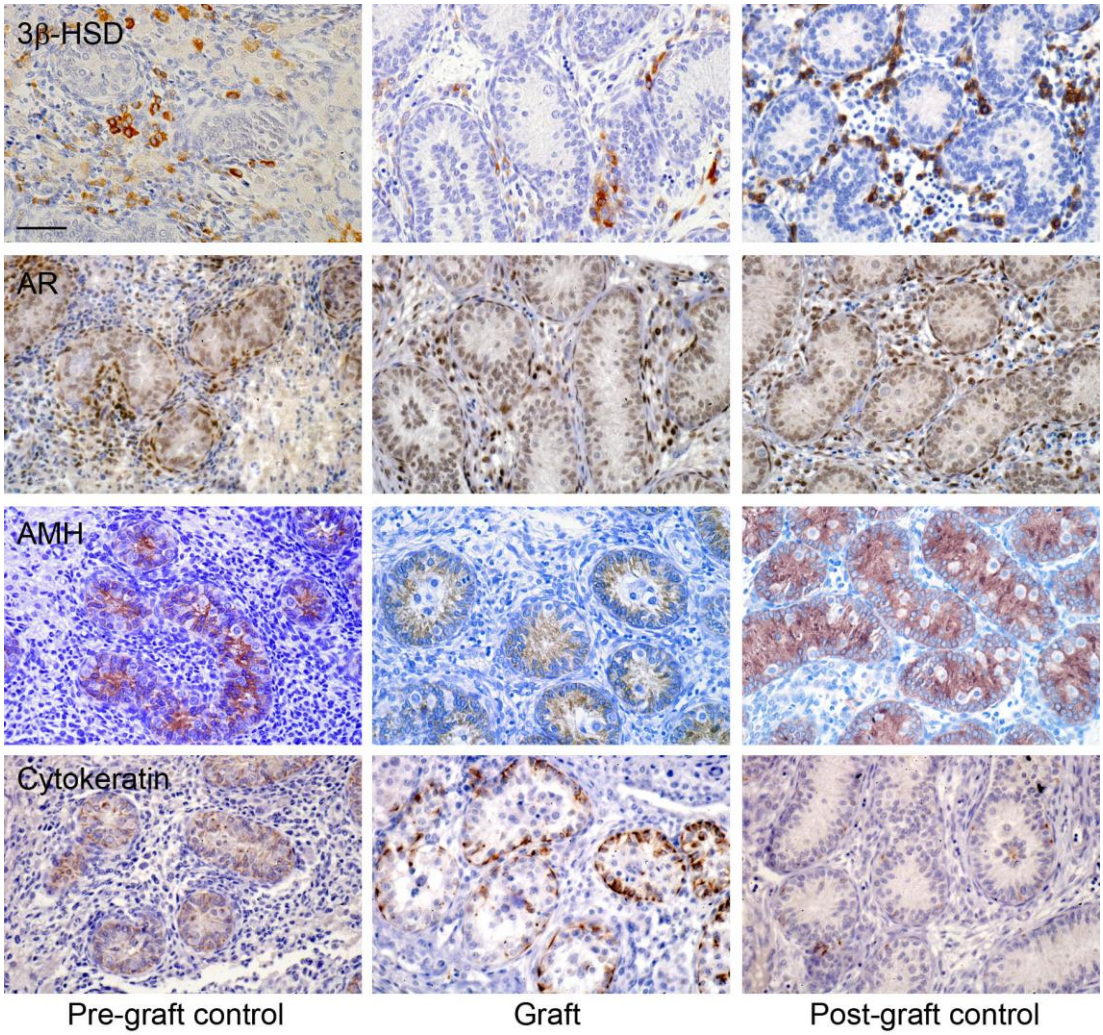

Figure 5.9. Expression of 3β-HSD, androgen receptor, AMH and pan cytokeratin in a xenograft of a neonatal marmoset testis. Grafts were retrieved after 6 weeks (middle panels). Comparison with testis from neonatal pre-graft tissue (left panels) and a six week old postnatal post graft control (right panels). Scale bar = 50μm.

There was evidence of germ cell differentiation in the grafts when compared to the controls. There was a reduction in the proportion of germ cells that expressed AP-2γ and an increase in the proportion that expressed MAGE-A4 (Figure 5.10). The same pattern of loss of early markers (OCT4) and expression of germ cell differentiation proteins (VASA) was also noted in co-staining experiments (Figure 5.10).

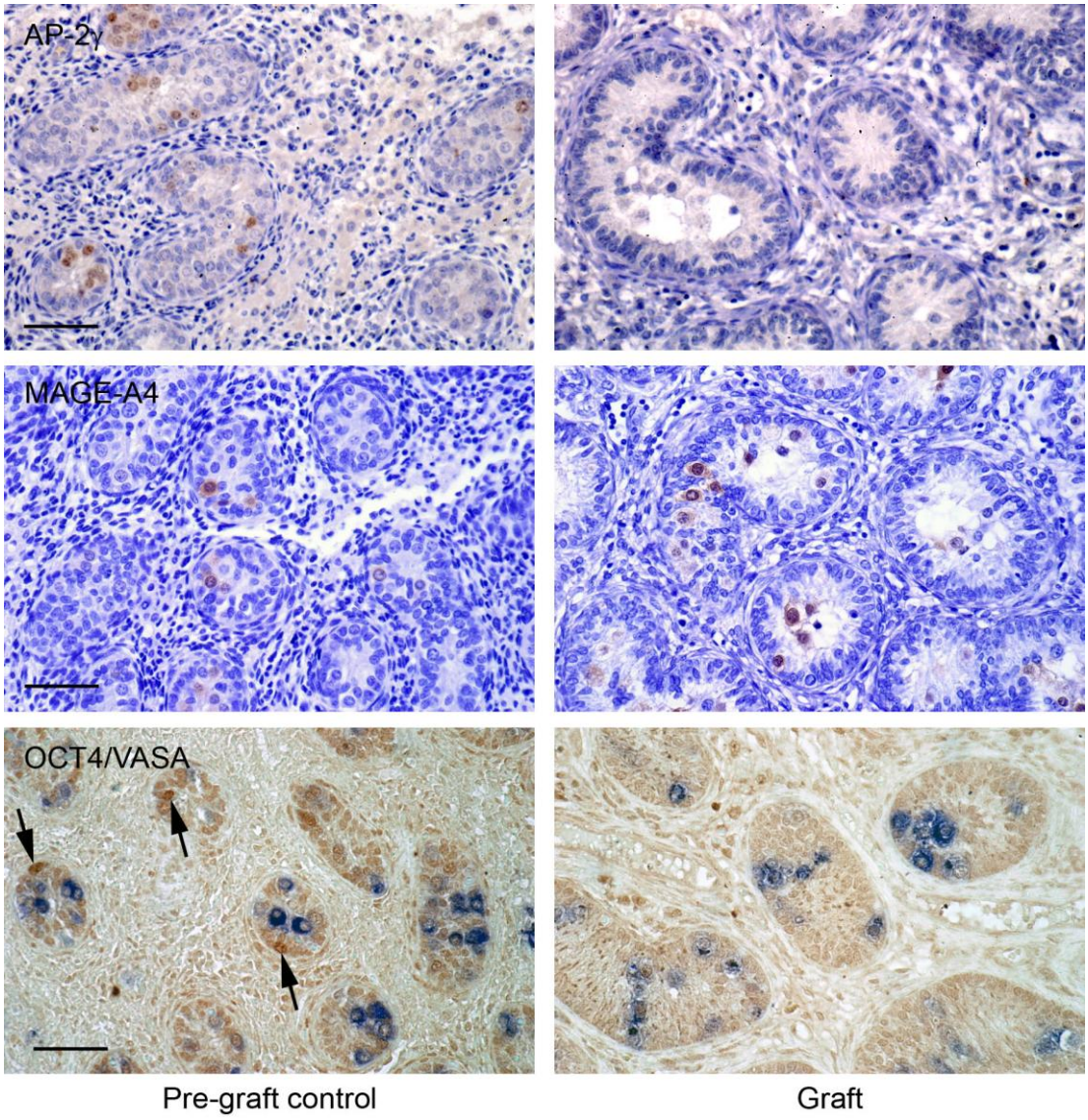

Figure 5.10. Expression of AP-2γ, MAGE-A4 and OCT4/VASA in neonatal marmoset testis xenografts. Grafts were retrieved after 6 weeks. Note the reduction in AP-2γ expression and increase in MAGE-A4 expression after grafting. OCT4 (brown) is also reduced (arrows) after grafting, whilst VASA (blue) expression increases. Scale bar = 50μm.

5.4.2.3. Juvenile marmoset testis xenografts

Germ cell development in the juvenile marmoset has been demonstrated to have similarities to the human (see Chapter 4). We grafted 25 week old marmoset testes for 10 weeks in order to assess the ability of these grafts to survive and differentiate/proliferate during this relatively quiescent 'childhood' period. In total, 24 xenografts were implanted and of these 58% were retrieved. All of these grafts contained seminiferous tubules with a qualitatively normal appearance. Of the four hosts into which tissue was grafted, 2 were castrate and 2 were intact. Castrated animals had small seminal vesicles and low serum testosterone levels similar to those obtained in untreated host animals in the previously described grafting results (Table 5.6). For each host larger grafts (2mm) were placed down the left side of the back, whilst grafts of standard size (1mm) were placed down the right side of the back. The larger grafts were more likely to be retrieved after the grafting period than the smaller ones and they were on average more than twice the weight of the smaller grafts (Table 5.7; Figure 5.11).

	Seminal vesicle (mg)	Serum testosterone (ng/ml)	Number of grafts	Grafts retrieved (%)	Mean graft weights (mg)
Castrate	23	0.16	6	50	3.3
Castrate	14	<0.1	6	67	1.1
Intact	240	8.29	6	50	1.1
Intact	333	1.26	6	50	1.7

Table 5.6. Effect of castration on seminal vesicle weight and serum testosterone of the four host mice receiving juvenile marmoset testis grafts.

Graft size	Number of grafts	Grafts retrieved (%)	Mean graft weights (mg)
1mm	12	33	0.88
2mm	12	83	1.95

Table 5.7. Effect of initial graft size on subsequent retrieval rates and graft weights in juvenile marmoset testis grafts.

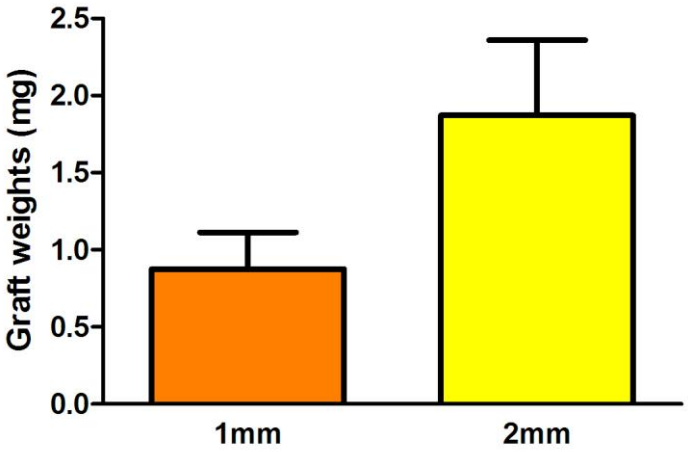

Figure 5.11. Graft weights for juvenile marmoset testis grafts. Original grafts were standard size (1mm) or larger size (2mm), n=12. Mean ± sem. p>0.05.

The seminiferous tubules of the grafts had a normal appearance with germ cells and Sertoli cells present. In addition the process of lumen formation was seen in some of the tubules (Figure 5.12)

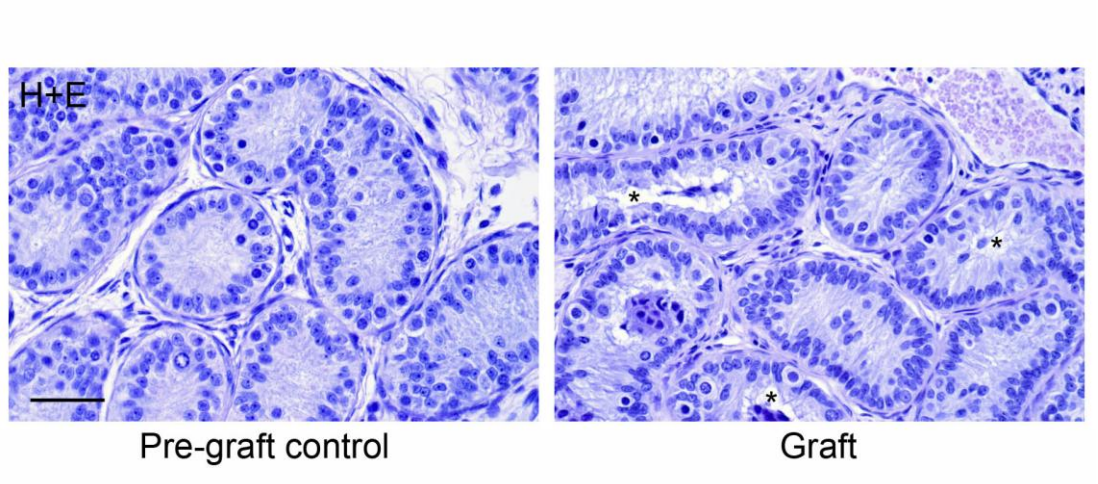

Figure 5.12. H+E. Juvenile marmoset testis xenografts. Testes from 25 week old postnatal marmosets were grafted for 10 weeks and compared with pre graft controls. Cord structure was maintained with germ cells and Sertoli cells clearly identifiable in the juvenile marmoset grafts (right panel) similar to pre-graft tissue (left panel). Lumen formation (*) can also be seen within the grafts. Scale bar = 50μm.

Both grafts and pre-graft controls expressed 3β-HSD in occasional interstitial cells. AMH was also expressed in both pre graft controls and grafts, with weak staining in both. Sertoli cells and peritubular myoid cells expressed AR (Figure 5.13)

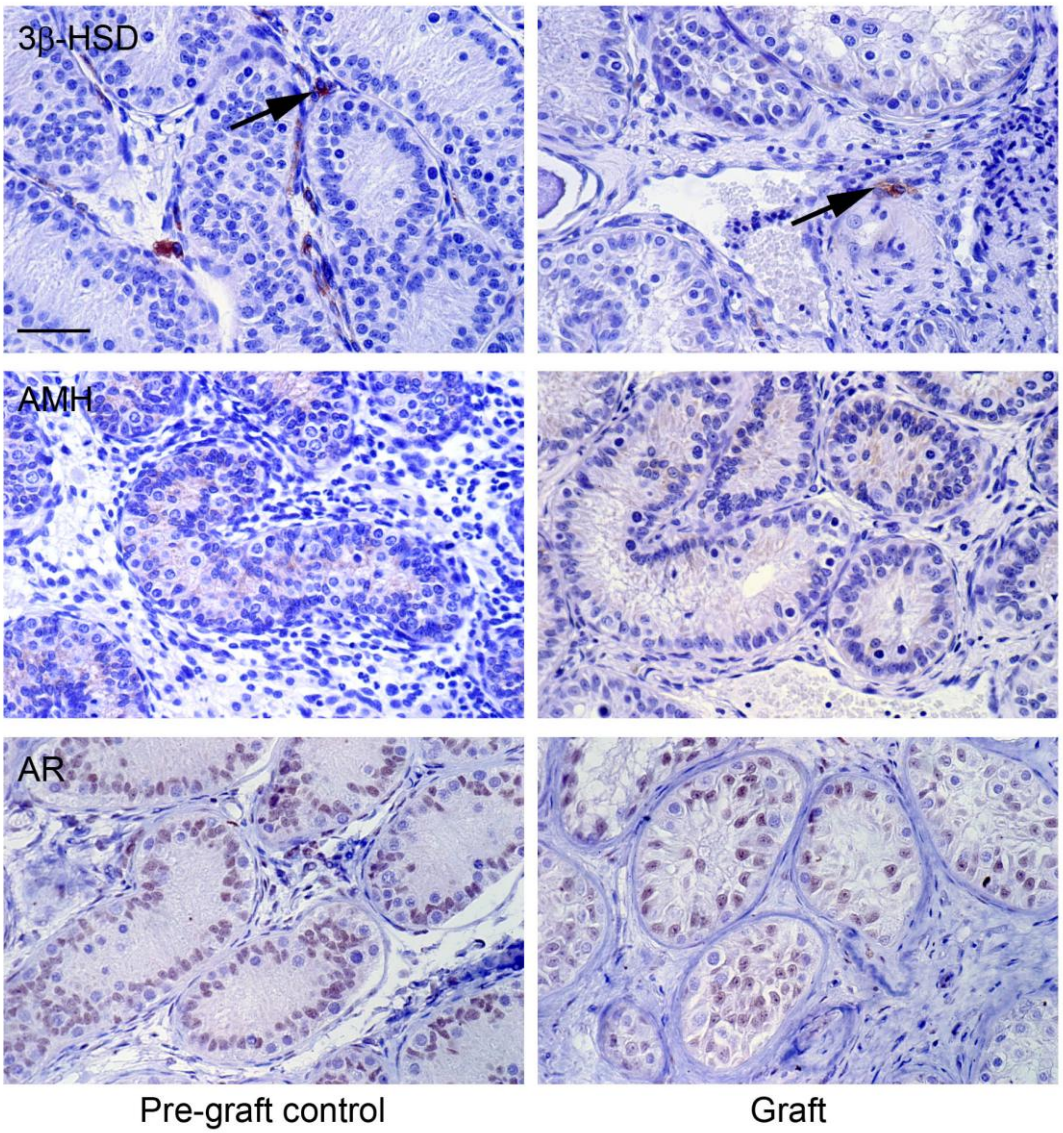

Figure 5.13. Expression of 3β-HSD, androgen receptor and AMH in a xenograft of a juvenile marmoset testis. Grafts were retrieved after 10 weeks. Note occasional Leydig cells express 3β-HSD (arrows). Comparison with testis from a 25 week pre-graft control. Scale bar = 50μm.

The grafts expressed markers characteristic of both pre and post-graft controls, such as VASA (Figure 5.14, also see Figure 4.16)

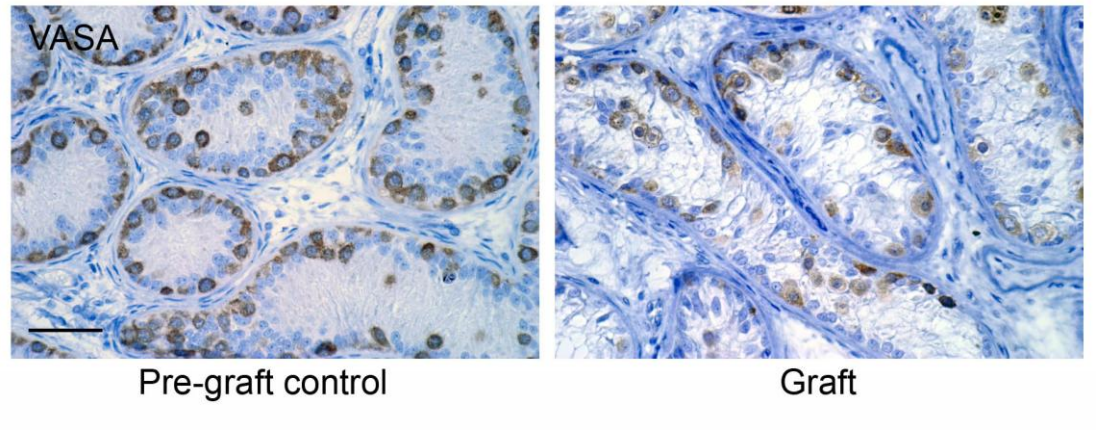

Figure 5.14. Expression of VASA in a xenograft of a 25 week old juvenile marmoset testis. Grafts were retrieved after 10 weeks and compared with testis from a 25 week pre-graft control. Scale bar = 50μm.

Germ cells within the juvenile marmoset grafts were proliferating as are those in the pre-graft control tissue. Proliferation appeared to occur in a higher proportion of germ cells compared to the pre-graft control (Figure 5.15).

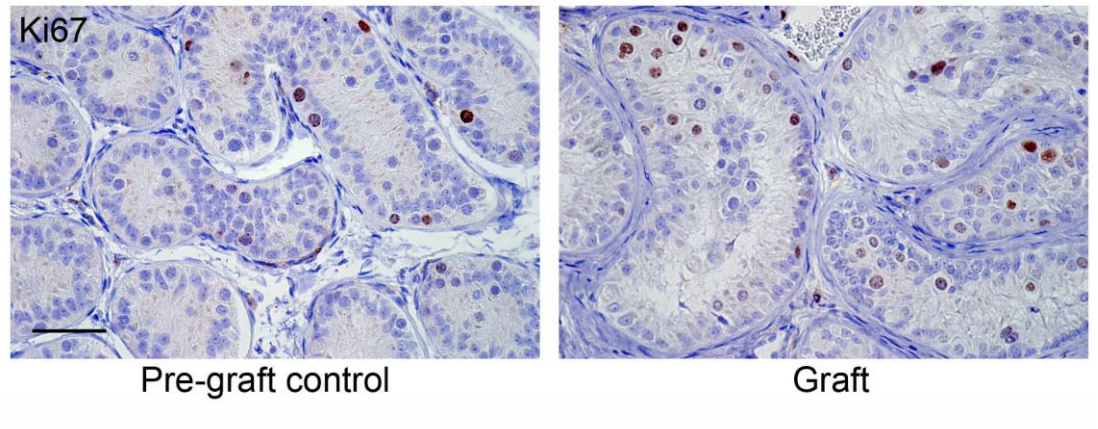

Figure 5.15. Expression of Ki67 in a xenograft of a 25 week old juvenile marmoset testis. Grafts were retrieved after 10 weeks and compared with testis from a 25 week pre-graft control. Scale bar = 50μm.

5.4.3. Human fetal testis xenografts

Having established an *in vivo* model of marmoset perinatal germ cell development we wished to investigate whether the technique would also be applicable to human fetal testis grafts, as this might have wide application. Testis grafts from both first and second trimester fetuses were grafted for 6 weeks.

5.4.3.1. Genotyping of first trimester fetal gonads

SRY genotyping was performed on tissue from each first trimester fetus in order to establish the sex of the fetus (Figure 5.16).

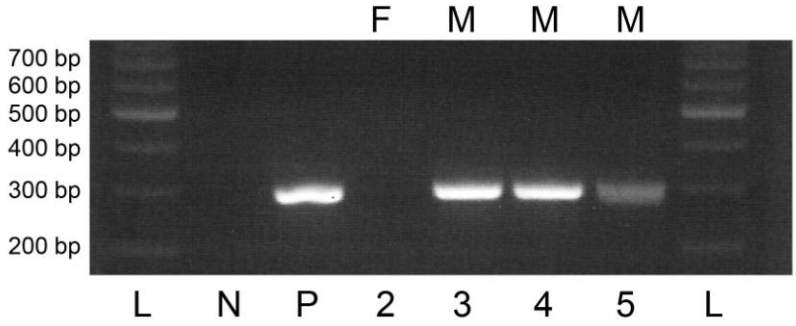

Figure 5.16. *SRY* genotyping in tissues from first trimester human fetuses. Note that 3/4 samples are (M) male and 1/4 are female (F). L = 100bp ladder, N = negative control, P = positive control. Product at 300bp.

5.4.3.2. First trimester human fetal testis xenografts

Grafts from four male first trimester (9 week gestation) fetuses were grafted for 6 weeks. The majority of the grafts were retrieved and all grafts had identifiable testis tissue (Table 5.8).

Age (weeks)	Grafting duration (weeks)	Grafts (total number)	Grafts retrieved (%)	Grafts with testis cords (%)
9	6	3	100	100
9	6	3	67	67
9	6	3	67	67
9	6	3	67	67
		12	**75**	**75**

Table 5.8. Success rate for the four first trimester fetal human testis xenografts.

The grafts from the first trimester testes did not produce detectable levels of serum testosterone and the mean seminal vesicle weight was 22mg (15-28), which was a similar value to that obtained for non-grafted castrate mice. The pre-graft control tissue had not completed seminiferous cord formation, however over the duration of the grafting period the seminiferous cords had fully formed with a normal appearance (Figure 5.17).

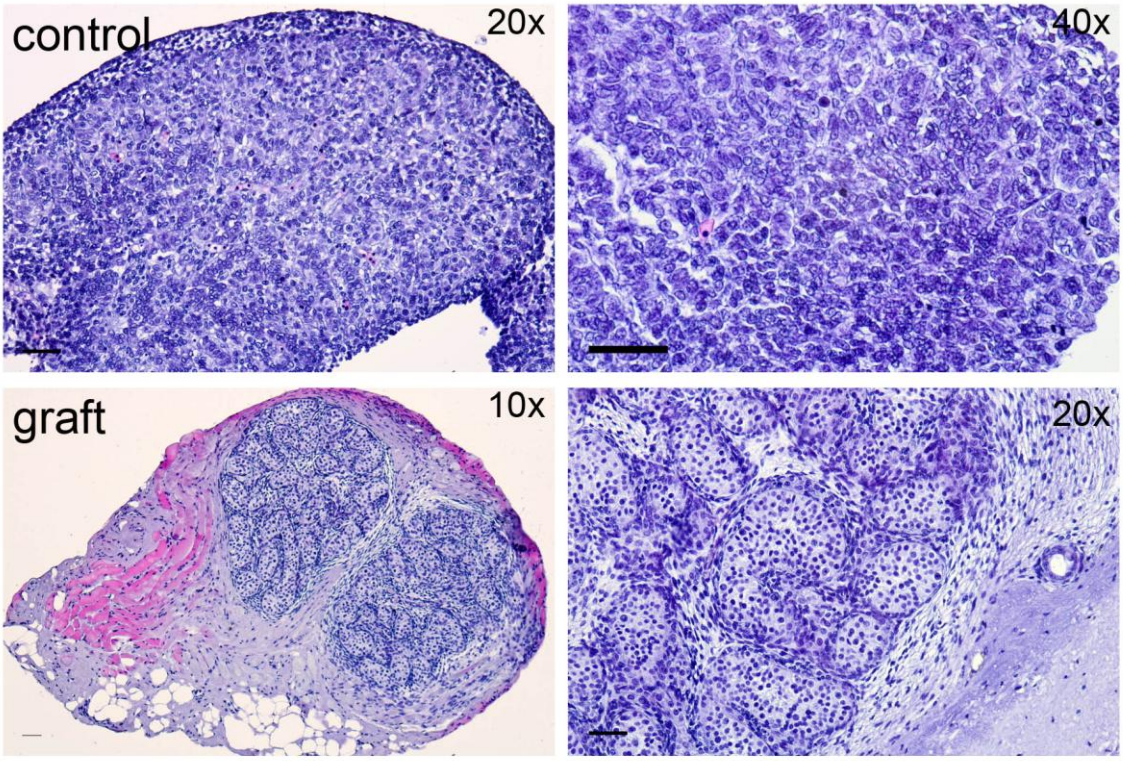

Figure 5.17. H+E. 9 week human fetal testis xenograft. Control testes lack completed seminiferous cord formation (upper panels), whilst grafts have developed cords with normal appearance (lower panels). Scale bar = 50μm.

In the pre-graft tissue the germ cells and Sertoli cells were distributed throughout the developing gonad. Many of the germ cells were not yet enclosed within identifiable testis cords although aggregates of Sertoli cells were apparent (Figure 5.18). However, 6 weeks after grafting seminiferous cords containing OCT4 expressing germ cells and SOX9 expressing Sertoli cells, had become more organised with the germ cells and Sertoli cells enclosed within clearly identifiable seminiferous cords outlined by staining with SMA in peritubular myoid cells (Figure 5.18).

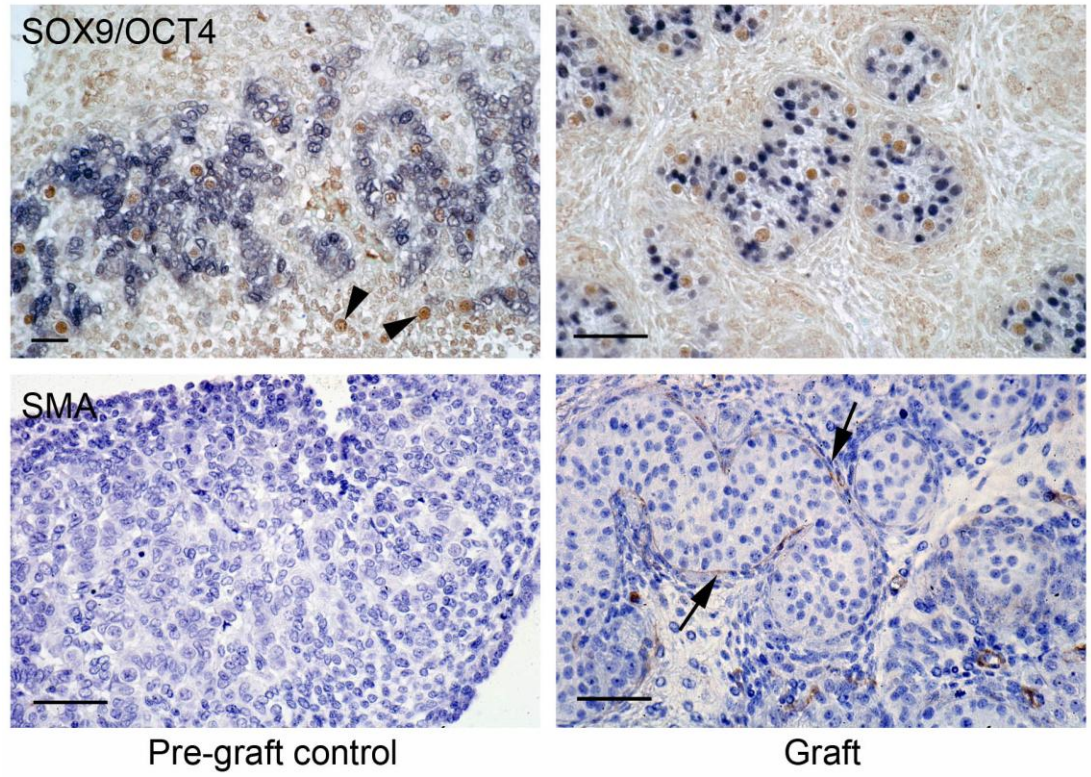

Figure 5.18. Expression of SOX9/OCT4 and SMA in first trimester human testis xenografts and controls. Isolated OCT4 (brown) germ cells can be identified (arrowhead) distant to the SOX9 (blue) Sertoli cells in controls, however all germ cells are enclosed within clearly defined seminiferous cords in grafts. The cords are outlined by the expression of SMA in the grafts (arrows), whilst the control tissue is negative for SMA. Scale bar = 50μm. Original magnification x40 (upper left panel x20).

The Leydig cells of the grafts were weakly immunopositive for 3β-HSD (Figure 5.19), indicating active steroidogenesis. The expression was not as strong as that of the equivalent post graft control, however no expression of 3β-HSD was demonstrated in the pre graft control. This indicates functional maturation of the Leydig cells during the grafting period in addition to the formation of the seminiferous cords. Peritubular myoid cells and some interstitial cells expressed AR, whilst Sertoli cells expressed AMH, which was similar to post-graft controls (Figure 5.19). Expression of these proteins was weaker in the pre graft controls than in the grafts.

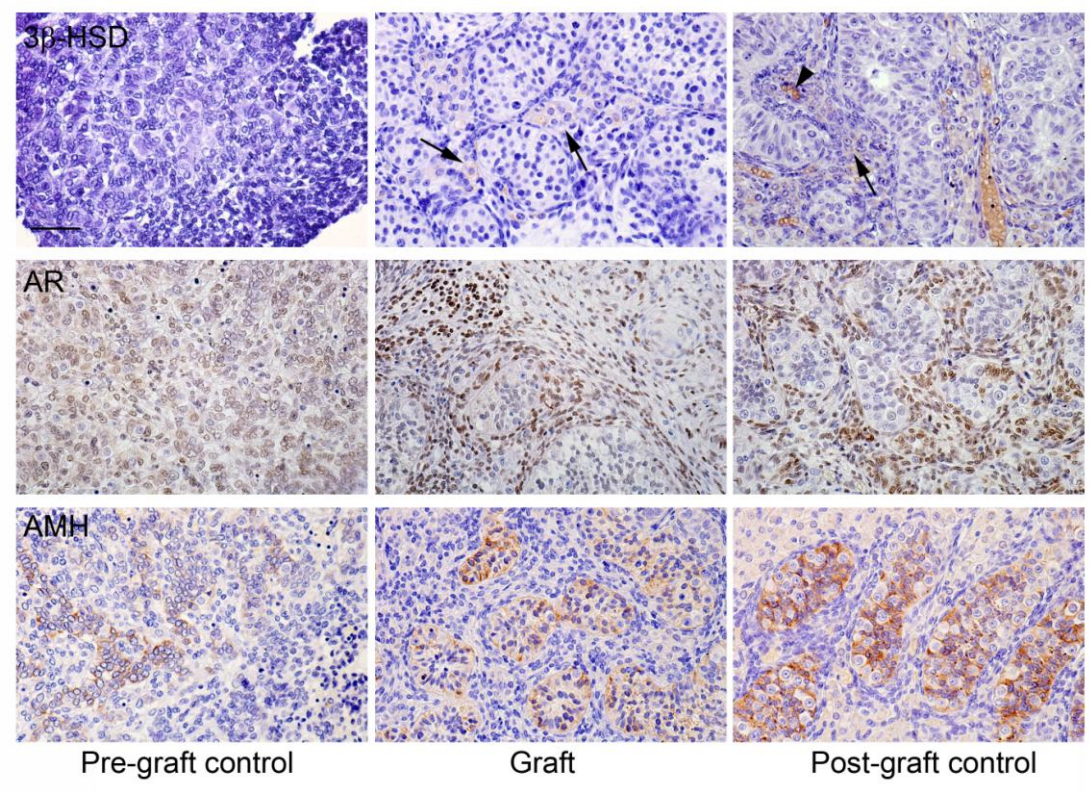

Figure 5.19. Expression of 3β-HSD, AR and AMH in a xenograft of a first trimester human testis. Grafts were retrieved after 6 weeks, and compared with testis from the pre and post-graft controls. The pre-graft control was negative for 3β-HSD, whilst grafts and post-graft controls expressed this enzyme (arrows). Note that some red blood cells (arrowhead) are also labelled with the 3β-HSD antibody in the post-graft control. Pre-graft controls had weak expression of AMH and AR compared to the grafts and post-graft controls. Scale bar = 50μm.

5.4.3.3. Second trimester human fetal testis xenografts

Grafts from four second trimester (14-17 week gestation) fetuses were grafted for 6 weeks. The majority of the grafts were retrieved and all grafts had identifiable testis tissue with testis cords exhibiting normal appearance in all but one of the retrieved grafts (Table 5.9).

Age (weeks)	Grafting duration (weeks)	Grafts (total number)	Grafts retrieved (%)	Grafts with testis cords (%)
14	6	18	88	72
15	6	36	67	67
16	6	5	80	80
17	6	6	100	100
		65	**77**	**72**

Table 5.9. Success rate for the four second trimester fetal human testis xenografts.

Serum testosterone and seminal vesicle weights indicated that the grafts were producing small amounts of testosterone. The testosterone production was much reduced compared to grafted, non-castrate mice at the equivalent age (Table 5.10).

	Testosterone		Seminal vesicle weight	
	ng/ml	range	mg	range
Testis grafts (castrate host)	0.13	0.1-0.26	20	17-28
Testis grafts (intact host)	3.4	n/a	225	n/a

Table 5.10. Serum testosterone and seminal vesicle weight for hosts receiving second trimester human fetal testis xenografts.

The seminiferous cords had a normal structure and they contained both germ cells and Sertoli cells with a visible basement membrane (Figure 5.20).

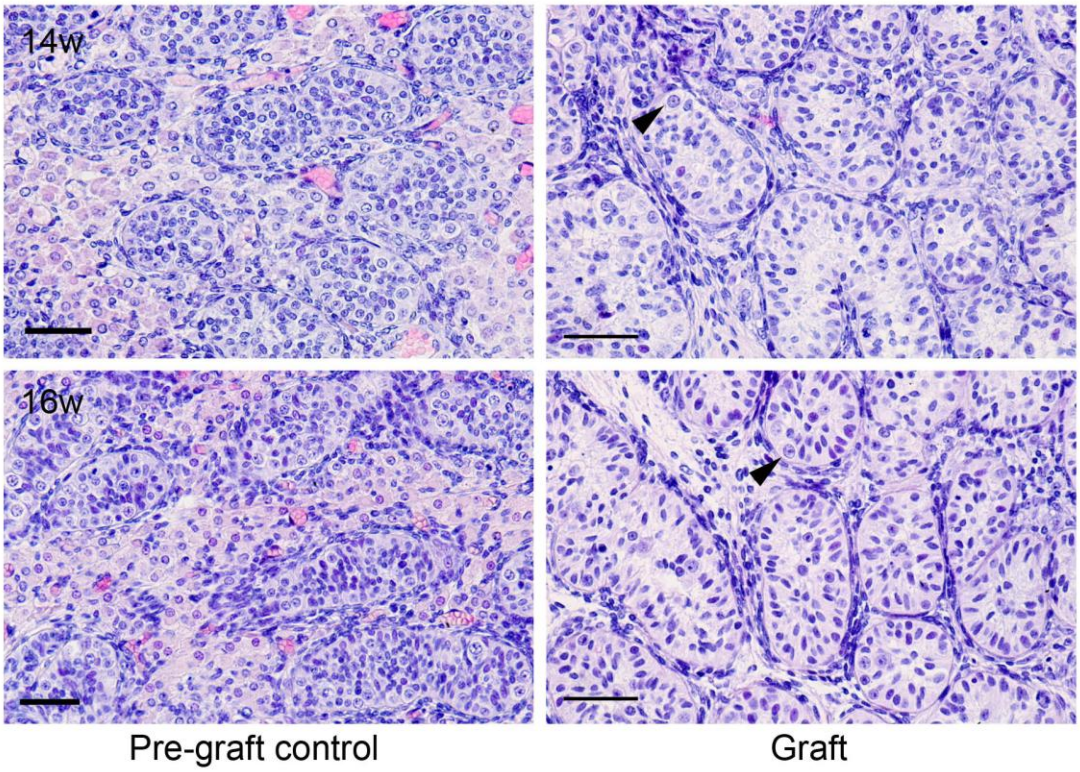

Figure 5.20. H+E. Second trimester human fetal testis xenografts. Cord structure was maintained with germ cells (arrowheads) and Sertoli cells clearly identifiable. Scale bar = 50μm. Original magnification x20 (left panels), x40 (right panels).

Some of the tissue from the 17 week human fetal testis was grafted into a nude mouse for an extended period of time. The grafts were left for 21 weeks, which would be equivalent to 38 weeks gestation (term), in order to see whether the capacity for germ cell survival was maintained. Of the 6 grafts, 5 (83%) were retrieved and all of the grafts exhibited preservation of normal cord structure (Figure 5.21). Germ cells were present throughout the tissue and were located in both central and basal positions.

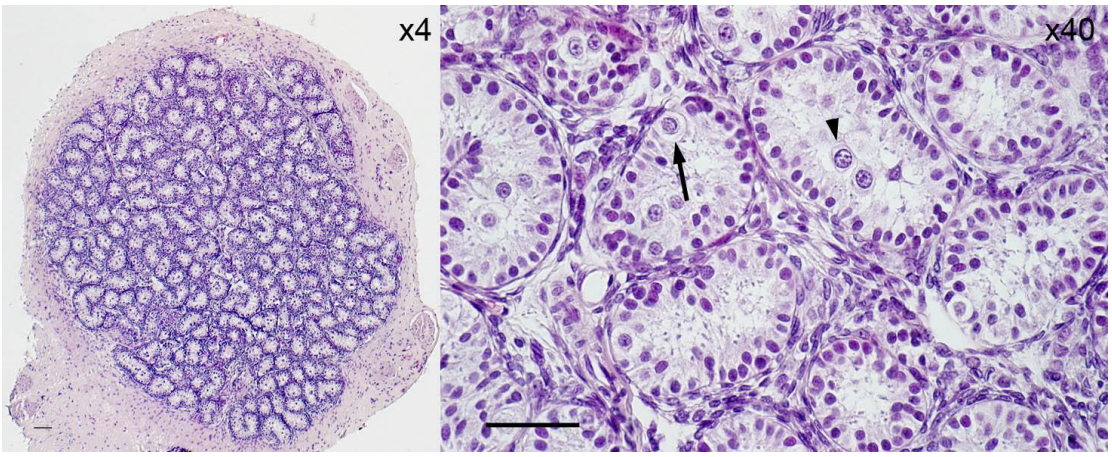

Figure 5.21. H+E. Second trimester (17 week) human fetal testis xenograft grafted for 21 weeks. Cord structure was maintained with basal (arrow) and central (arrowhead) germ cells, and Sertoli cells clearly identifiable. Scale bar = 50μm. Original magnification x4 (left panel), x40 (right panel).

AR and AMH were expressed in second trimester grafts after 6 weeks and the equivalent post graft controls (Figure 5.22). This expression was similar to that of the equivalent pre-graft controls (See post-graft control, Figure 5.19). AMH expression in the post-graft control was less intense than expected.

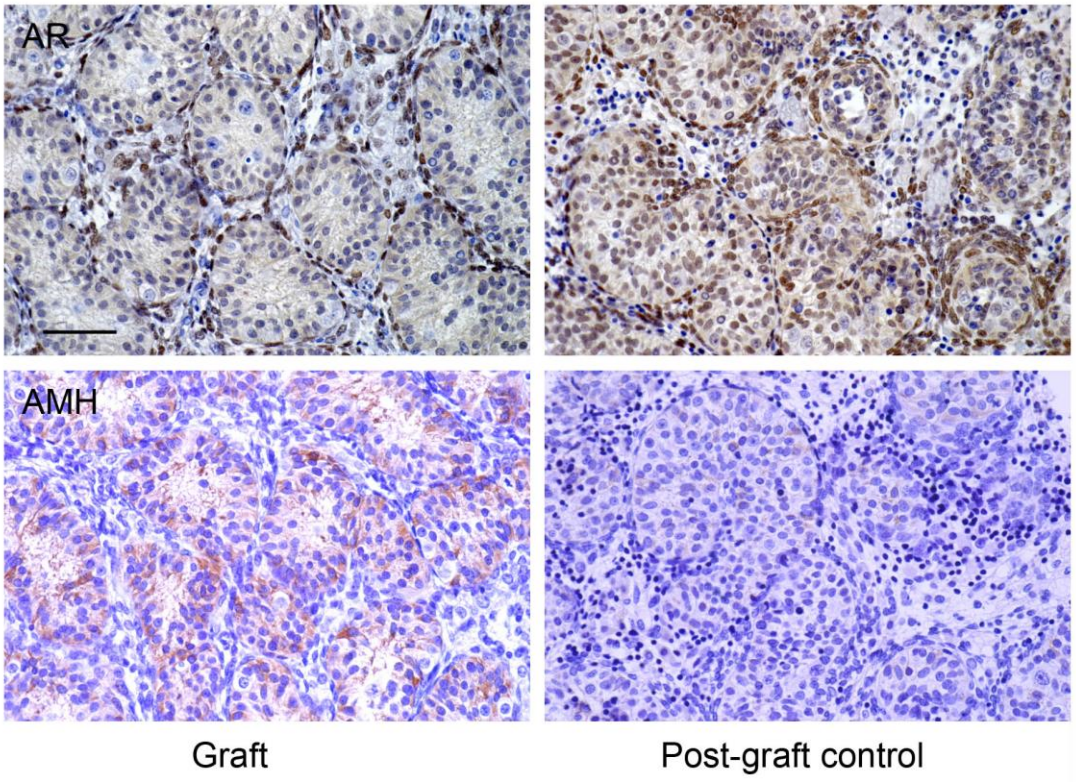

Figure 5.22. Expression of AR and AMH in a xenograft of a second trimester human fetal testis. Grafts were retrieved after 6 weeks and compared with testis from equivalent post-graft controls. Both grafts and post-graft controls expressed these markers, however AMH was weak in the post graft control. Scale bar = 50μm.

5.4.3.4. Testosterone production by human fetal testis xenografts and stimulation with hCG

Castration of the host animal resulted in cessation of testosterone production from the host animal. The aim was to increase the host gonadotrophin levels to stimulate testosterone production from the grafts and enhance growth and development of the grafts. However as the levels of testosterone were much lower than in intact animals the responsiveness of the graft Leydig cells to exogenous hCG was assessed.

Testosterone was undetectable in animals grafted with first trimester tissue. Hosts grafted with second trimester tissue also had undetectable or very low levels of

testosterone. However hCG treatment of hosts with second trimester xenografts resulted in a significant increase in serum testosterone and seminal vesicle weight (Figure 5.23).

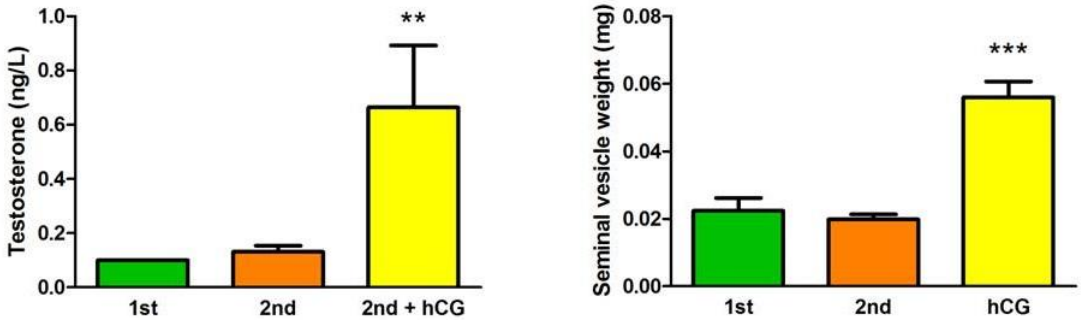

Figure 5.23. Testosterone production and seminal vesicle weights in untreated first (1st) and second (2nd) trimester human fetal testis xenografts and the effects of hCG on second trimester grafts. n=3. Mean ± sem. Statistical analysis performed using ANOVA. ** p<0.01, *** p<0.001.

An increase in graft weight was observed in the hCG treated animals carrying second trimester grafts compared to the vehicle treated controls, but this increase was not statistically significant (Figure 5.24).

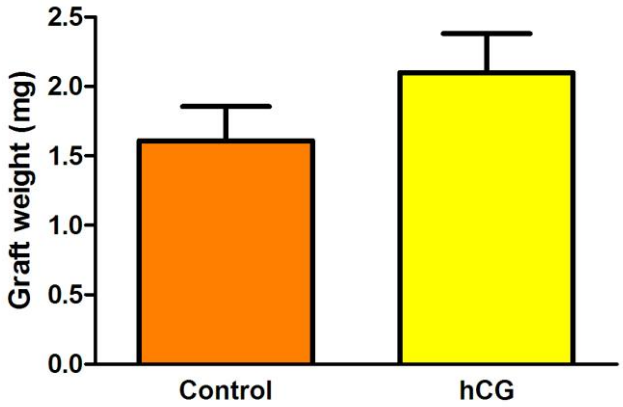

Figure 5.24. Graft weights in control and hCG treated grafts from second trimester human fetuses. (n=3). Mean ± sem. Statistical analysis performed using unpaired students t-test. p>0.05.

In addition to the increase in host serum testosterone level and seminal vesicle weight, histological examination of grafts demonstrated that there was hypertrophy of Leydig cells within the hCG treated grafts (Figure 5.25). These enlarged Leydig cells tended to be more prominent around the periphery of the grafts. In untreated and vehicle treated grafts the Leydig cells appeared to be involuted (Fig. 5.25)

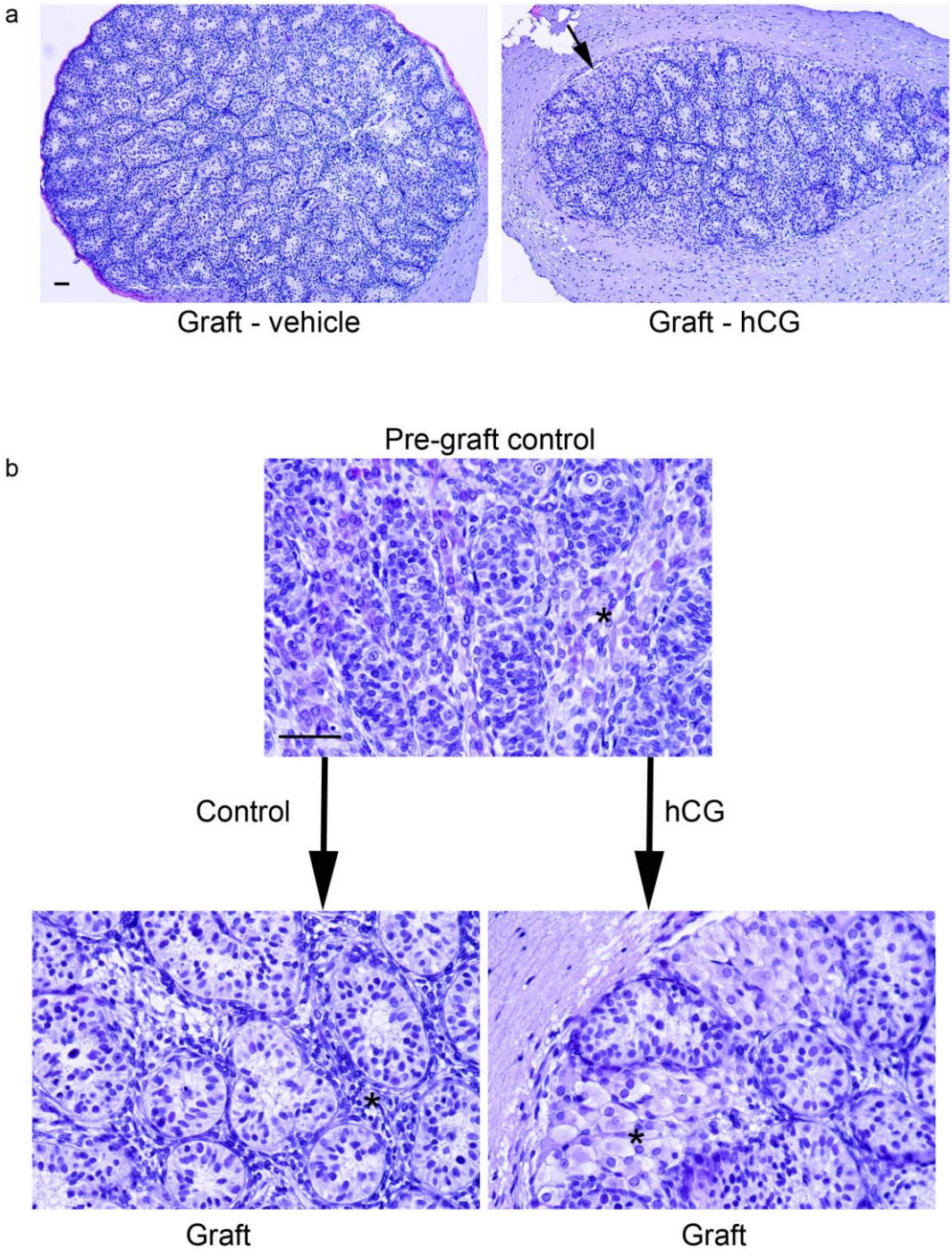

Figure 5.25. H+E. Effect of hCG treatment on second trimester human fetal testis grafts. a) Vehicle treated and hCG treated grafts. Leydig cell hyperplasia was noted at the periphery of the exposed grafts (arrow). Original magnification x10. b) Comparison of Leydig cells(*) size and distribution in pre graft controls, untreated grafts and hCG treated grafts. Scale bar = 50μm.

There was also a difference in expression of 3β-HSD in grafts from second trimester human fetuses in which the hosts had been treated with hCG, compared with untreated controls (Figure 5.26). hCG treated grafts expressed 3β-HSD, whilst most of the vehicle treated grafts were immunonegative for this enzyme.

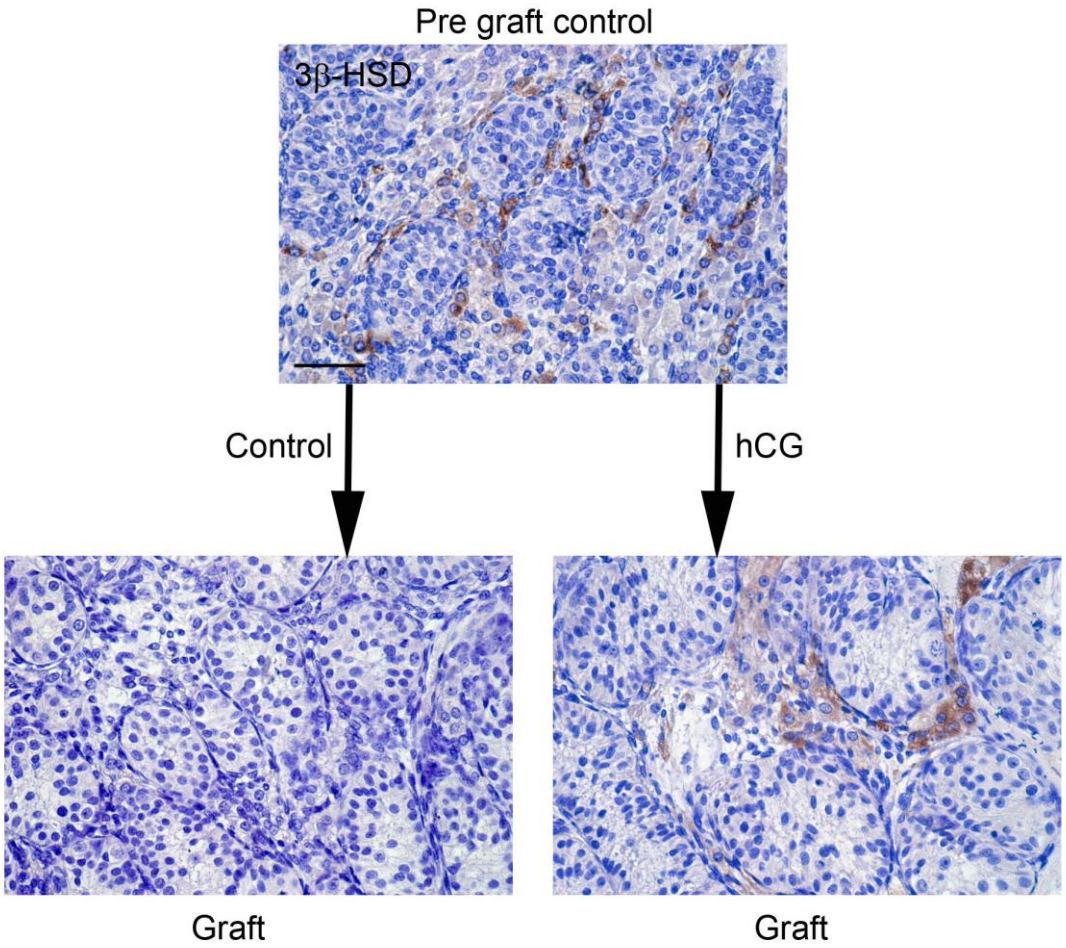

Figure 5.26. Effect of hCG treatment on 3β-HSD expression in second trimester human fetal testis grafts. Scale bar = 50μm.

5.4.3.5. Germ cell development in human fetal testis xenografts

Germ cell development in human fetal testis xenografts was investigated by calculating the germ cell to Sertoli cell ratio in addition to analysis of germ cell differentiation. The germ cell to Sertoli cell ratio was not significantly different between grafts and controls for either first or second trimester grafts (Figure 5.27).

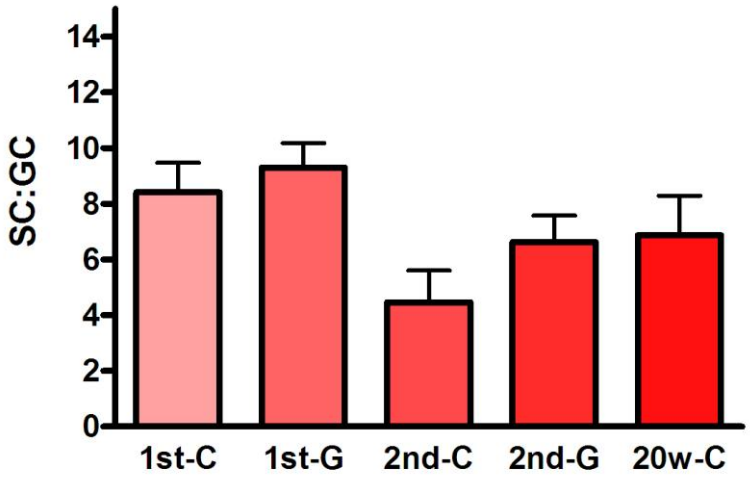

Figure 5.27. Germ cell (GC) to Sertoli cell (SC) ratio in human fetal testis xenografts. Number of Sertoli cells per germ cell in first (1st) and second (2nd) trimester human fetal testis xenografts (G) and controls (C), (n=3, 20w ctrl n=2). Mean ± sem. p>0.05. w=weeks.

There was evidence of differentiation of germ cells in the human fetal testis xenografts. Germ cells in first trimester control testes expressed OCT4, whilst VASA and MAGE-A4 were almost completely absent from germ cells at this age. OCT4 expression was seen in a lower proportion of germ cells in grafts compared to pre-graft controls. The reduction in the proportion of cells expressing OCT4 coincided with the appearance of expression of VASA and MAGE-A4 (Figure 5.28).

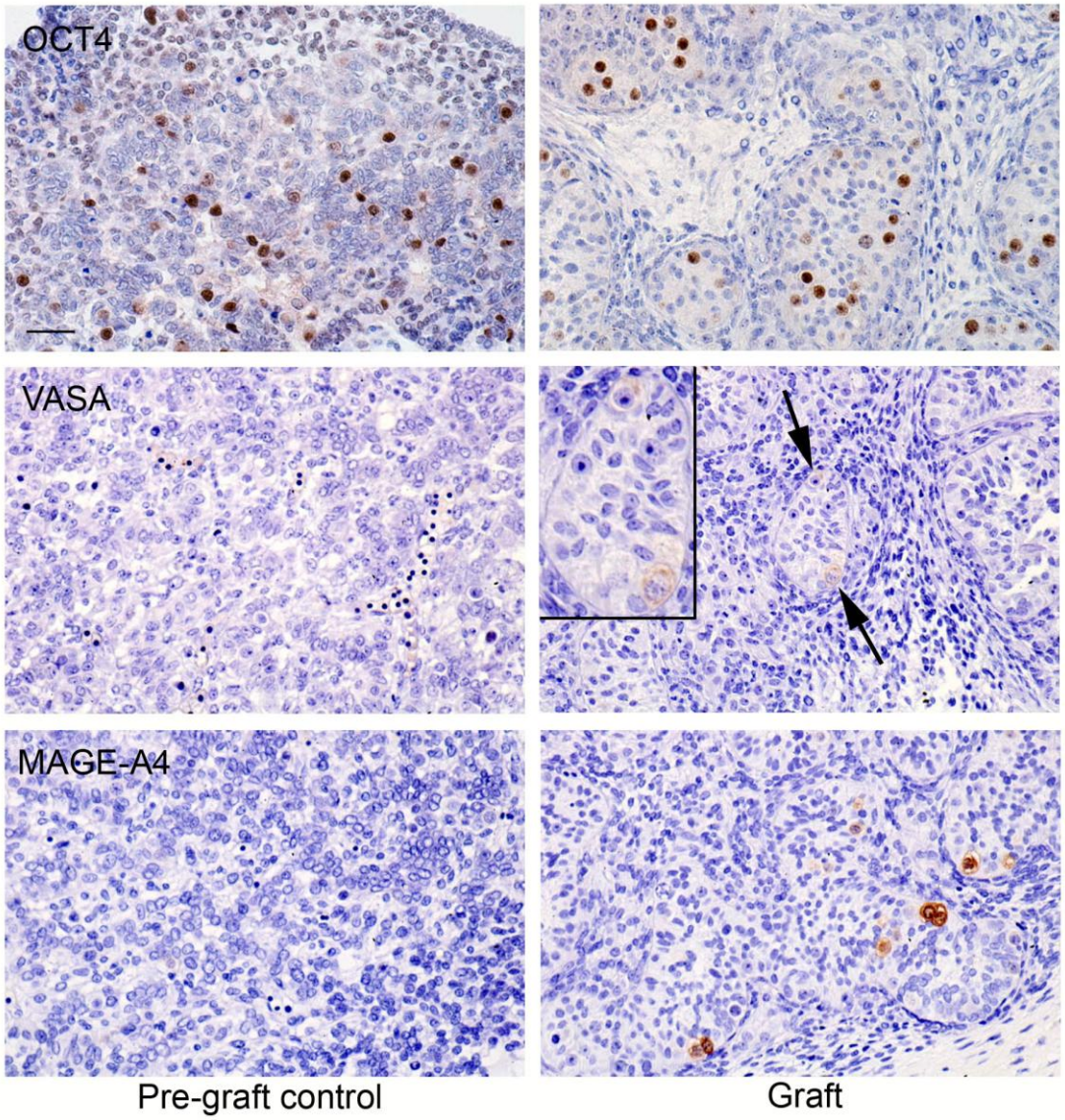

Figure 5.28. Expression of OCT4, MAGE-A4 and VASA in a xenograft of a first trimester human testis. Grafts were retrieved after 6 weeks. Note the reduction in OCT4 expression with increasing VASA (arrows) and MAGE-A4 expression after grafting. Scale bar = 50μm.

In second trimester grafts, the proportion of germ cells expressing OCT4 decreased further compared to the pre-graft control, whilst the proportion of germ cells expressing VASA increased and the intensity of MAGE-A4 increased (Figure 5.29)

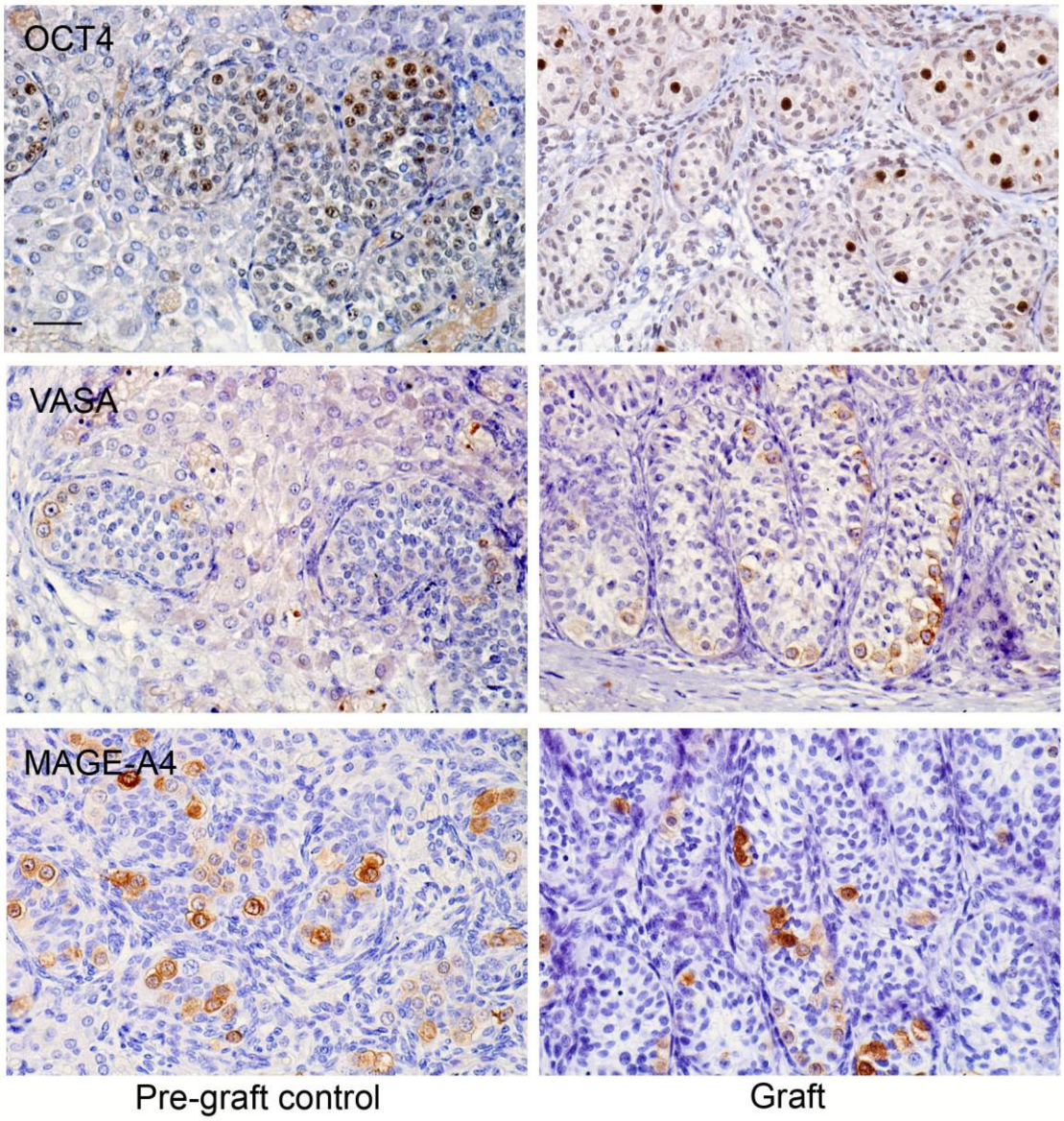

Figure 5.29. Expression of OCT4, MAGE-A4 and VASA in a xenograft of a second trimester human fetal testis. Grafts were retrieved after 6 weeks. Note the reduction in OCT4 expression with increasing VASA and MAGE-A4 expression after grafting. Scale bar = 50μm.

The change in expression of the germ cell differentiation markers was quantified by co-localising OCT4 with either VASA (not shown, example of OCT4/VASA staining is shown in Figure 5.7b) or MAGE-A4 (Figure 5.32). With both approaches the proportion of germ cells expressing OCT4 decreased, whilst the proportion

expressing VASA or MAGE increased with increasing equivalent age of control or graft (Figure 5.30). The proportions of germ cells expressing these markers were not significantly different between the grafts and the equivalent post graft control. In addition the proportions were not different between the second trimester grafts treated with hCG when compared to the vehicle treated second trimester grafts. The proportion of germ cells expressing both markers was less than 10% for both OCT4/VASA and OCT4/MAGE-A4 and did not differ between any of the groups (Figure 5.30).

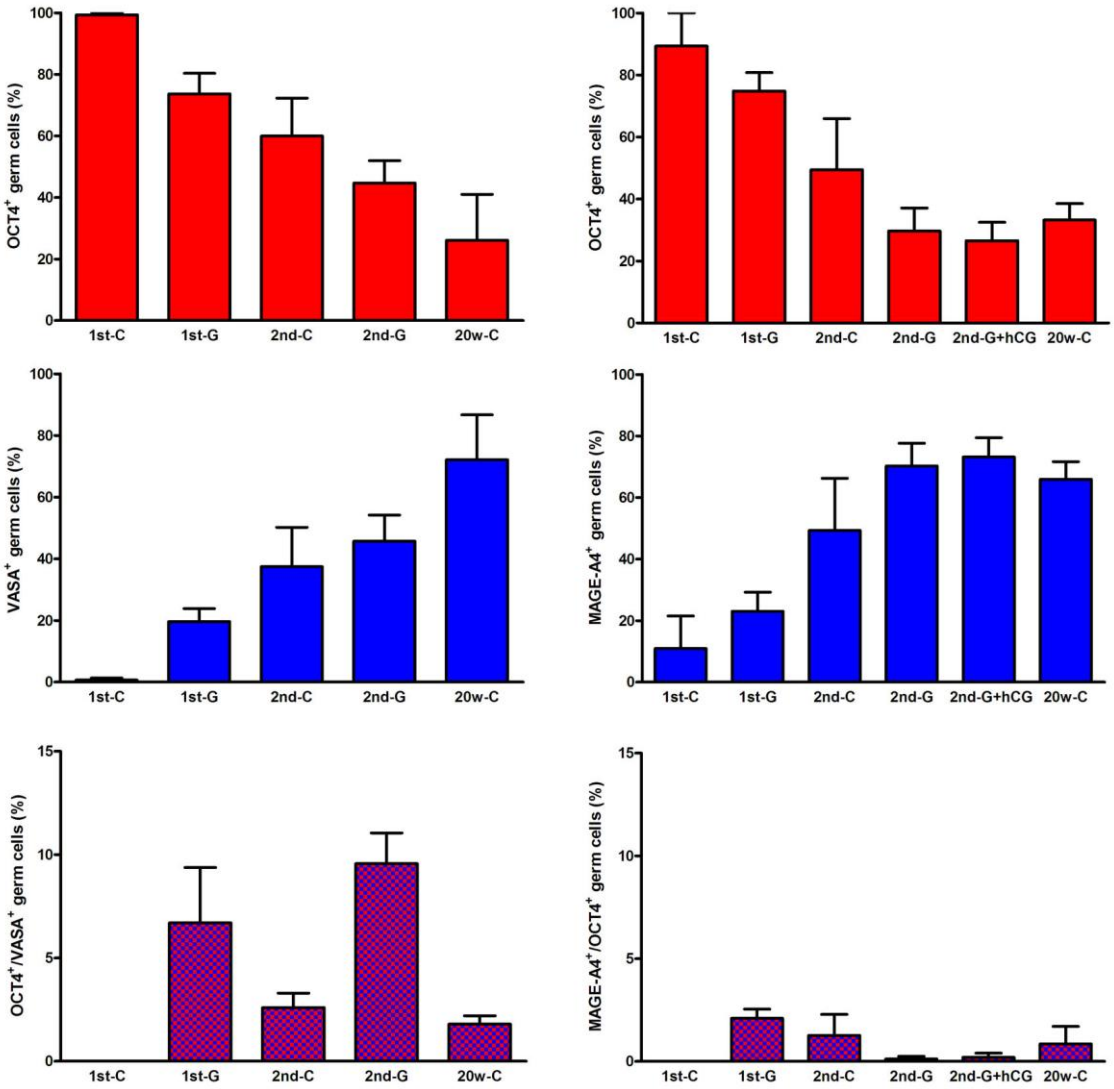

Figure 5.30. Germ cell differentiation in human fetal testis xenografts. Proportion of cells expressing OCT4, VASA or OCT4 + VASA (left panels) and OCT4, MAGE-A4 or OCT4 + MAGE-A4 (right panels) in first (1^{st}) and second (2^{nd}) trimester human fetal testis xenografts (G) and controls (C). n=3 (20w ctrl, n=2). Mean ± sem. w=weeks.

5.4.3.6. Proliferation of germ cells in human fetal testis xenografts

Germ cells and Sertoli cells were proliferating in the human testis xenografts. Mitotic germ cells were seen within the grafts in addition to germ cells positive for Ki67 (Fig. 5.31).

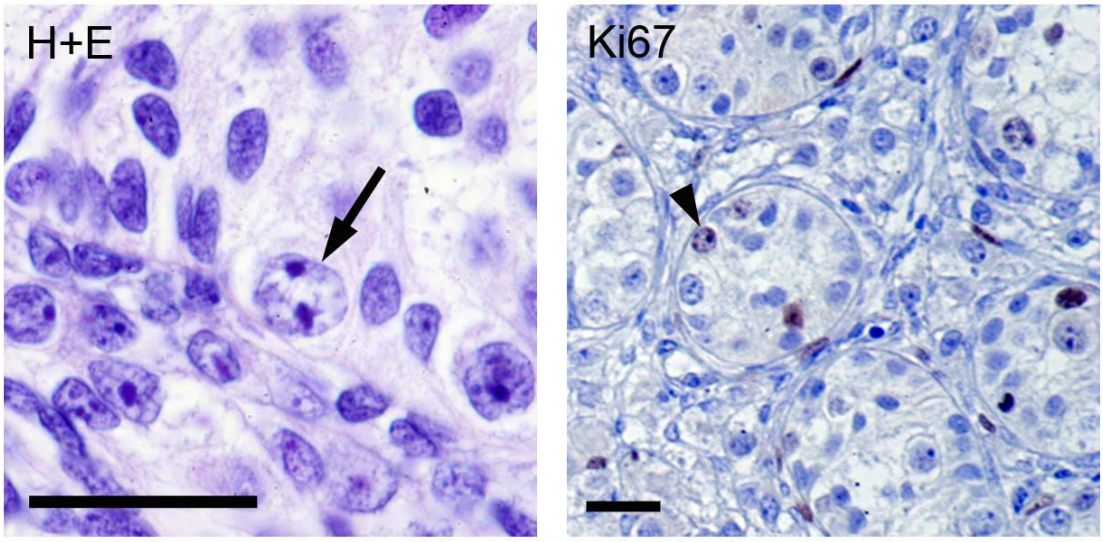

Figure 5.31. Proliferation of germ cells from a 14 week human fetal testis xenograft. Grafts were retrieved after 6 weeks. Mitotic germ cells (arrow) can be seen on H+E sections. Ki67 was also expressed in some of the germ cells (arrowhead). Scale bar = 25μm

Triple staining of OCT4/MAGE-A4/Ki67 demonstrated that a proportion of all three subpoulations of germ cells ($OCT4^+$/$MAGE\text{-}A4^-$, $OCT4^+$/$MAGE\text{-}A4^+$ and $OCT4^-$/$MAGE\text{-}A4^+$) were proliferating (Figure 5.32).

It was also apparent that the proliferation rate differed between these subpopulations. The proliferation index was quantified in these subpopulations within the grafts and compared them to those of controls (Figure 5.33). It could be seen that the OCT^+/$MAGE\text{-}A4^-$ cells were the most proliferative, whilst the subpopulations that expressed MAGE-A4 had very low rates of proliferation. This was the case for both first and second trimester grafts and the same pattern was also found in the pre and post-graft controls. No significant differences were seen between the proportions of proliferating subpopulations of germ cells within grafts or controls, with the exception of OCT4 expression in the second trimester grafts and the equivalent post graft control (Figure 5.32).

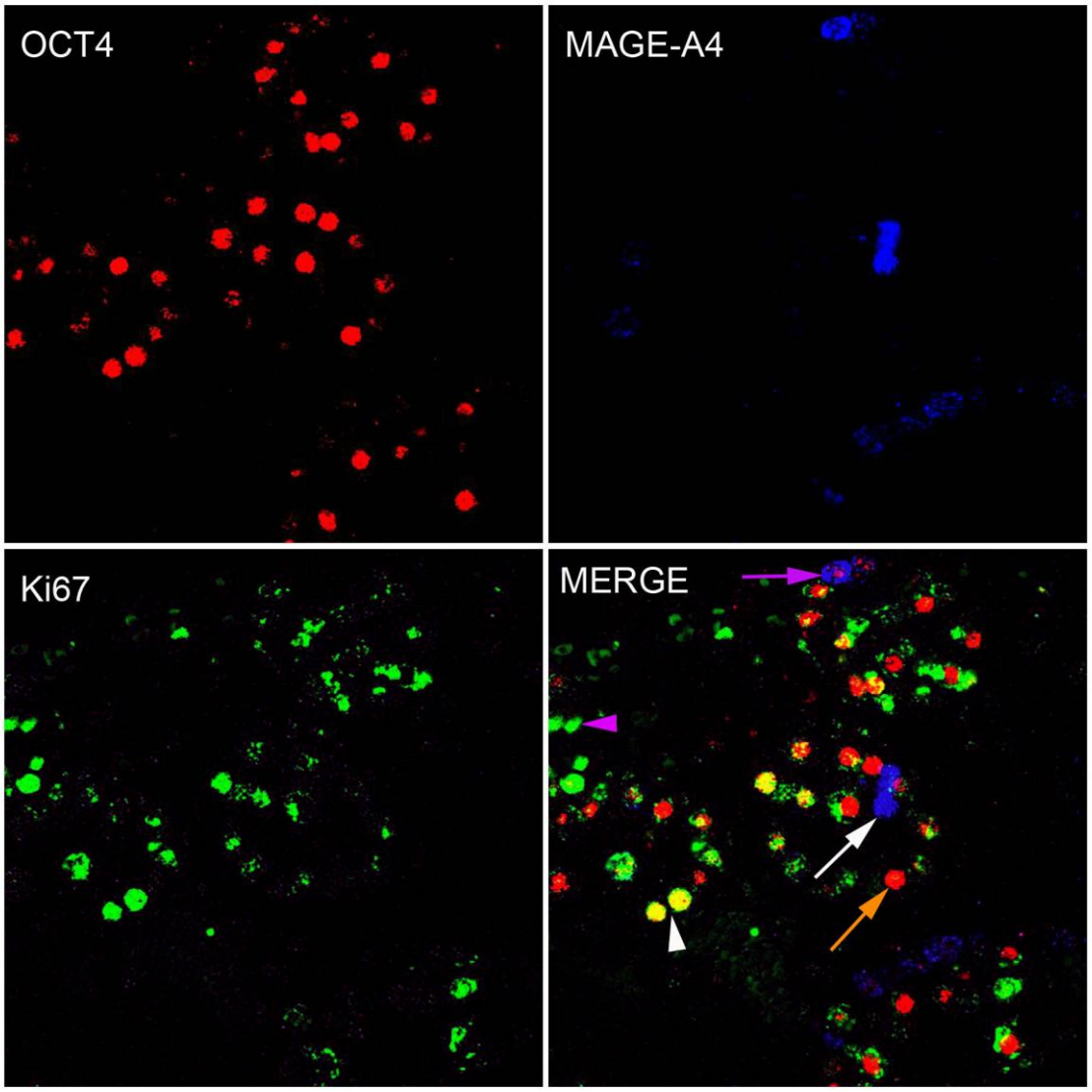

Figure 5.32. Proliferation in the subpopulations of germ cells in a second trimester human fetal testis xenograft. Expression of OCT4 (red), MAGE-A4 (blue) and Ki67 (green) in a 14 week gestation human fetal testis xenograft. Note the expression of $OCT4^{+}$/$MAGE\text{-}A4^{-}$ (orange arrow) $OCT4^{+}$/$MAGE\text{-}A4^{+}$ (purple arrow) and $OCT4^{-}$/$MAGE\text{-}A4^{+}$ (white arrow) germ cells and that some of the $OCT4^{+}$/$MAGE\text{-}A4^{-}$ cells are proliferating (white arrowhead). The cells stained for Ki67 that are negative for OCT4 and MAGE-A4 represent proliferating Sertoli cells (purple arrowhead).

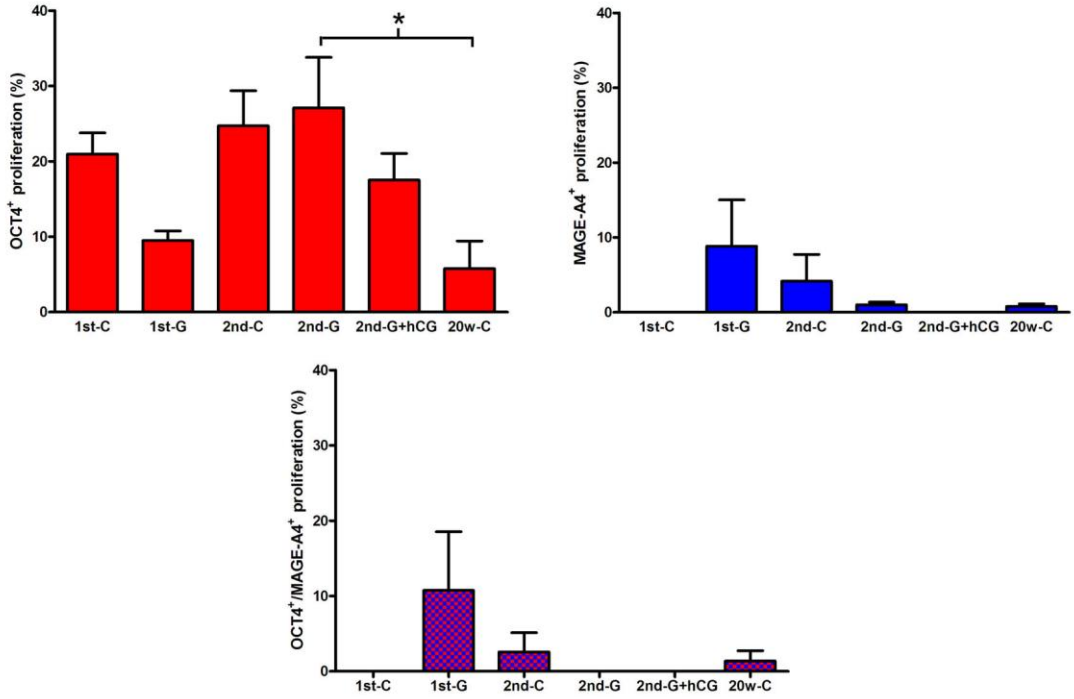

Figure 5.33. Germ cell proliferation in the subpopulations of human fetal testis xenografts. Proportion of proliferating cells expressing OCT4, MAGE-A4 or OCT4 + MAGE-A4 in first (1^{st}) and second (2^{nd}) trimester human fetal testis xenografts (G) and controls (C). n≥3. Mean ± sem. * p<0.05.

5.5. Discussion

5.5.1. Testicular xenografts show excellent survival and provide large numbers of grafts

This study has demonstrated that testicular xenografts from fetal and postnatal animals of various species show good survival over a period of several weeks. Our results for neonatal mouse allografts compare favourably with previous studies. We demonstrated 100% recovery of mouse grafts after 4 and 8 weeks of grafting compared with 100% at 4 weeks and 97% at 8 weeks in another recent study (Yu et al., 2006). An earlier study had reported a graft recovery at 4 weeks of 60% (Schlatt et al., 2003);(Honaramooz et al., 2002). The proportion of retrieved grafts that contain recognisable testis tubules in our study was 72%, which compared favourably with 65% in one of the previous studies (Schlatt et al., 2002). In addition

these grafts progressed to completion of spermatogenesis as has been described by other groups (Schlatt et al., 2003);(Honaramooz et al., 2002);(Yu et al., 2006). This validated our grafting protocol for use in other species.

Fetal marmoset grafts have not been reported previously, however in the present studies, retrieval rates (90%) and the presence of seminiferous tubules within grafts (79%) compares very favourably with the rates described for human fetal testis grafts and in comparison to immature testis grafts in other species (Yu et al., 2006);(Schlatt et al., 2003);(Honaramooz et al., 2002). In our study, survival of fetal human xenografts ranged from 67 to 100%. Overall 75% of human fetal grafts were retrieved after 6 weeks with recognisable seminiferous tubules in 72%. This compared with 50% retrieval (all of which had recognisable tubules) between 16 and 19 weeks of grafting in a previous study (Yu et al., 2006).

For neonatal marmoset xenografts we retrieved 100% of grafts and 72% had demonstrable seminiferous tubules, which is similar to previous reports in neonatal marmoset testis grafts retrieved after 12 weeks (Wistuba et al., 2004). In juvenile marmoset grafts, the retrieval rate was lower than in fetal and neonatal grafts with only 58% of grafts retrieved, all of which had recognisable seminiferous tubules. A higher retrieval rate of 78% was found in a study of infant rhesus monkeys (Rathi et al., 2008). This is consistent with the concept that in a variety of species, increasing age and maturity of the donor tissue results in less successful recovery of tissue (Kim et al., 2007);(Schlatt et al., 2002);(Geens et al., 2006);(Rodriguez Sosa and Dobrinski, 2009);(Arregui et al., 2008b). This is particularly the case for grafting of adult testes in which meiosis has begun. The reason for this reduced success may be related to the cell complement within the seminiferous tubules as they mature, or alternatively may be due to an increased susceptibility of mature tissue to ischaemia (Rathi et al., 2006).

5.5.2. Testis xenografts from mouse, human and marmoset produce testosterone

Functional activity of the Leydig cells within the testis xenografts from each of the species was examined by measurement of serum testosterone and seminal vesicle weight. Castration of host mice prevented production of host testosterone, which meant that measured testosterone and seminal vesicle weights were an accurate bioassay of androgen produced by the grafts (Schlatt et al., 2002). Seminal vesicle weight showed good correlation with serum testosterone levels. Testosterone levels varied between tissue donor age and species, with highest levels obtained using mouse grafts and lower levels using human and marmoset grafts. Serum testosterone levels were even variable between animals that received grafts from the same donor, which may reflect the number and size of the surviving grafts and the amount of functional Leydig cell activity within the grafts, although it is recognised that testosterone levels can be inherently variable between mice (Schlatt et al., 2002). Neonatal marmoset grafts did not produce testosterone, which is consistent with previous reports (Schlatt et al., 2002);(Wistuba et al., 2004). This had been hypothesised to be due to several reasons such as the difference in the LH receptor in the marmoset which is not responsive to mouse LH, although administration of CG (to which the marmoset LH receptor is responsive) did not improve androgen production in the grafts (Wistuba et al., 2004). This led to the hypothesis that an unknown species specific factor is required for successful grafting in the marmoset. It is interesting to note that in the present study the fetal marmoset grafts were able to produce low levels of testosterone and this suggests that such a species specific reason for failure of neonatal grafts to produce testosterone is unlikely. It is therefore possible that there may be an age related factor that prevents these grafts from producing androgens.

5.5.3. Testosterone production can be increased in human fetal testis xenografts by the administration of hCG

Although neonatal marmoset xenografts do not respond to exogenous hCG, the fact that the fetal marmoset and fetal human grafts produced low levels of testosterone led us to speculate that the Leydig cells of the human fetal testis xenografts may respond to hCG and increase testosterone production. Administration of hCG resulted in a significant increase in the serum testosterone levels and seminal vesicle weights in the host mice that had received second trimester human fetal testis xenografts and the graft weights were increased in these animals, compared to vehicle treated controls. Effects of hCG on the Leydig cells themselves was demonstrated by hypertrophy of the Leydig cells in the grafts from hCG treated hosts. Expression of 3β-HSD also occurred in the grafts from mice that received hCG, whilst grafts retrieved from non-hCG treated mice did not express this steroidogenic enzyme. This demonstrates that the Leydig cells are functional and inducible, which may be important in the context of further studies that investigate the effects of various treatments on androgen production in these grafts. It has also been suggested that the lack of production of androgens may be responsible for the failure of gonadal differentiation in the neonatal marmoset (Wistuba et al., 2004) and therefore the induction of increased levels of testosterone could result in enhanced differentiation in the human fetal testis grafts.

5.5.4. Seminiferous cords can form *in vivo* using the testis xenografting technique

This study has demonstrated that xenografts from first trimester human fetal testes in which organisation of the testis cords is incomplete can complete cord formation following subcutaneous xenografting into nude mice. This is a novel finding and has not previously been demonstrated using testis material from any other species. However it is interesting to note that dissociated cells from rodent testes have been used to re-form testicular cords both *in vitro* (Gassei et al., 2006) and *in vivo*

(Honaramooz et al., 2007);(Honaramooz et al., 2007);(Kita et al., 2007);(Arregui et al., 2008a). In these experiments the cells are dissociated and either grown in 3D culture systems and subsequently transplanted into host mice (Gassei et al., 2006), or alternatively transplanted directly into nude mice (Honaramooz et al., 2007);(Kita et al., 2007). These studies involved the use of testis tissue in which the cords have already formed prior to their dissociation. To date this technique has not been performed using human tissue or testis from any species prior to cord formation, or using pre-cord formation testis tissue. This technique has tremendous potential use for mixing different combinations of germ, Sertoli and Leydig cells and looking at the effect of altering the characteristics of the individual cells within the mixture on the development of the testis. This could also be applied to the first trimester human fetal testis in which the cords have not fully developed to recapitulate initial cord formation and increase understanding of how the seminiferous cords form in the human and how dysgenesis might arise or be induced.

5.5.5. Structure and cell complement in testis xenograft

The xenografts from all ages and species exhibited good preservation of testis structure. The grafted testis tissue was enclosed within a fibrous capsule and contained interstitial cells, including Leydig cells. The seminiferous tubules had a normal appearance with a basement membrane and contained germ cells, Sertoli cells and peritubular myoid cells, which can all be identified by immunoexpression of protein markers. Some of the grafts demonstrated central degeneration and in most of the grafts the tubules and Leydig cells tended to exhibit better preservation at the periphery of the grafts rather than centrally. In sheep xenografts, tubules containing spermatogonia were more frequent in the periphery of the grafts, whilst tubules in the centre contained SCO tubules (Arregui et al., 2008a). Proliferation was retarded until the blood supply had been restored (Rathi et al., 2005) and this may be the reason for the variability between tubules in different areas of the graft. Diffusion, prior to the establishment of a blood supply may result in improved

development at the periphery of the tubule, compared to central regions (Arregui et al., 2008a). Germ cells were present in the majority of cords and the ratio of germ cells to Sertoli cells was quantified for human fetal testis xenografts. The ratio of Sertoli cells to germ cells in the first trimester controls and grafts was 8:1 and 10:1 respectively, which is similar to previous reports for normal human fetal testes (Ostrer et al., 2007);(Bendsen et al., 2003). There was no significant difference between controls or grafts, suggesting that there is no imbalance between the survival of the two cells types, however it is not known whether the total number of germ cells and Sertoli cells is changed with the grafting technique. Quantification of total germ cell number is difficult due to the fact that the grafts may also contain surrounding skin and muscle as well as the testis tissue, therefore deriving 'testis' weight and volume is challenging. It is likely that there is some initial loss of germ cells initially after grafting, but the numbers may recover in some cases over the grafting period. Indeed we have obtained evidence of proliferation of both Sertoli and germ cells within the grafts. Germ cell proliferation in second trimester human fetal testis xenografts, as demonstrated by Ki67 straining, was occurring in 20-30% of cells. In grafts from second trimester fetal testis there was a significantly higher germ cell proliferation rate than in the equivalent post graft control. This may indicate that there is a compensatory increase in germ cell proliferation in response to germ cell loss, or alternatively that these cells are simply continuing to behave like the pre-graft control. Further studies will be required if we are to accurately determine the germ cell and Sertoli cell numbers. The present study investigated proliferation but not apoptosis. Further work is required to determine the relative contribution of proliferation and apoptosis to germ cell numbers.

5.5.6. Germ cell development in testis xenografts

Neonatal mouse testis xenografts achieved full spermatogenesis and displayed a full complement of germ cells, as has been described in previous studies (Schlatt et al., 2002);(Honaramooz et al., 2002);(Yu et al., 2006). Xenografts from human and

marmoset testes contained a mixture of germ cells. This included germ cells that were located near the basement membrane and had the appearance of spermatogonia, whilst occasional germ cells with a gonocyte phenotype were identified in the centre of some cords. Cells with the same appearances were also identified in grafts from the second trimester human fetal testes in previous studies, however further characterisation of these germ cells was not performed (Yu et al., 2006);(Povlsen et al., 1974);(Skakkebaek et al., 1974). We investigated the identity of the germ cells within the grafts further by looking at the expression profiles of germ cell proteins described previously in chapters 3 and 4. In grafts from human fetal testes, we observed differentiation of germ cells following a similar pattern to normal germ cell differentiation. Expression of proteins such as VASA and MAGE-A4 was not detected in most first trimester testes (Gaskell et al., 2004);(Mitchell et al., 2008), however expression of these proteins was apparent in some of the germ cells after grafting. This is clear evidence that germ cells are able to differentiate in testis xenografts. In addition the proportions of germ cells with an undifferentiated 'gonocyte' (AP-2γ, OCT4) phenotype decreased in second trimester xenografts, whilst germ cells with a more differentiated 'spermatogonial' phenotype (VASA, MAGE-A4 expression) increased, compared to the pre-graft control. This has been demonstrated to occur in the normal process of fetal human germ cell differentiation (Gaskell et al., 2004);(Mitchell et al., 2008);(Honecker et al., 2004). In addition there was no significant difference in the proportions of cells expressing the individual phenotypes of germ cell differentiation between grafts and control testes. This included the identification of a transition population of germ cells that displayed an intermediate phenotype of co-staining of undifferentiated and differentiated germ cell markers. This suggests that there is no arrest of differentiation during this transition phase. Normal progression of germ cell differentiation has also been shown in testis xenografts for fetal and neonatal marmosets. The marmoset has already been shown to be a good model for germ cell development in Chapter 4 (Mitchell et al., 2008)) and therefore the use of marmoset

testis xenografts may provide an alternative model, which negates the use of human tissue. Previous studies of xenografting have demonstrated accelerated maturation of germ cells and spermatogenesis in some species occurring earlier than the equivalent time in the normal animal (Schmidt et al., 2006b), including in the primate (Honaramooz et al., 2004). In the current study there was no evidence of premature entry into meiosis in the human fetal grafts or marmoset fetal and postnatal grafts. However this is unsurprising given the relatively short grafting period.

5.5.7. Juvenile marmoset xenografts survive and retain spermatogonia

The fact that fetal and neonatal marmoset xenografts produce little or no testosterone may affect the development of these grafts, because if the tissue was in situ we would expect concentrations of testosterone to be high. Xenografts from more mature animals tend to result in a decrease in graft survival (Rodriguez Sosa and Dobrinski, 2009), and it has been suggested that this is because of the presence of mature germ cell types (Huang et al., 2008). However, juvenile primate testes do not contain meiotic germ cells and normal testis development and germ cell proliferation at this age does not require high levels of testosterone (Plant, 2006);(Kelnar et al., 2002). Very few studies have been performed using pre-pubertal testes for grafting (Oatley et al., 2005);(Goossens et al., 2008);(Honaramooz et al., 2002). Completion of spermatogenesis has been demonstrated in juvenile rhesus monkey grafts (Rathi et al., 2008) and therefore we wished to investigate whether the juvenile marmoset may be suitable for testis xenografting, given that germ cell development in this species is very similar to the human. Retrieval rates were lower for these grafts than for the fetal and neonatal grafts. In addition the production of testosterone and seminal vesicle weights were also low. Testis structure and the appearance of the seminiferous cords appeared similar to controls during the 10 week grafting period and there was characteristic staining for markers of germ, Sertoli, Leydig and peritubular myoid cells. In addition numerous germ cells

expressing VASA were identified within the grafts, similar to pre- and post-graft controls and we were able to show that spermatogonia were able to continue to proliferate. Although quantification was not performed, it appeared that there was a higher proportion of proliferating germ cells in the grafts compared to the pre-graft control. This could be due to an increase in proliferation to compensate for loss of germ cells immediately after grafting, or alternatively it is possible that the proliferating germ cells survive the grafting process better than the quiescent cells. Spermatogonial stem cells rarely divide and GFRα1 has been shown to be a marker of spermatogonial stem cells in testes of rodents (Buageaw et al., 2005). GFRα1 may also represent an SSC marker in rhesus monkeys (Hermann et al., 2007);(Hermann et al., 2009). Unfortunately the present study was unable to identify a reliable antibody for GFRα1 and therefore could not demonstrate the preservation of SSC in these grafts, although such information would be useful to determine the potential for subsequent spermatogenesis. There was no evidence for the germ cell development indicative of spermatogenesis in these grafts although they were still at a pre-pubertal equivalent age. However, there was initiation of lumen formation in the grafts similar to that seen in the post-graft controls (Kelnar et al., 2002). Testis xenografting using pre-pubertal human testes has produced similar results, with demonstration of survival of xenografts from two patients with sickle cell anaemia aged 10 and 11. Survival rates were 66% after grafting for 4 and 9 months and there were occasional surviving germ cells that expressed MAGE-A4 (Goossens et al., 2008). Survival of spermatogonia has also been shown in testes xenografts that were obtained at biopsy, from 5 boys aged 7-14 years and grafted for 6 months (Wyns et al., 2008). In addition, this study also demonstrated proliferation of cells within the surviving xenografts, based on Ki67 staining. Similar results were obtained from xenografts of testis tissue from young cryptorchid boys (Wyns et al., 2007). Successful xenografting of tissue taken from the juvenile age range could have future clinical relevance in terms of fertility preservation in childhood cancer survivors (Goossens et al., 2008);(Jahnukainen et al., 2006).

5.5.8. Conclusion

Testis xenografting provides a unique opportunity to recapitulate normal development of the testis in an *in vivo* system. The present studies have demonstrated that this is applicable to the study of fetal and neonatal development in the marmoset and also for fetal development in the human. The system can be used to produce numerous grafts of developing testis tissue from species in which it is difficult to perform direct interventions. This is particularly important for primate and human studies in which tissue supply, cost, logistics and acceptability of studies that assess treatment response are prohibitive. We have shown that human and marmoset grafts can reproduce testis development and this includes the first *in vivo* model for formation of the testicular cords. The opportunity to recapitulate testis development and the potential to manipulate the testicular environment may be used in the study of disorders of sex development, which occur during initial gonad formation, and the origins of CIS of the testis, which occurs during fetal life. The next chapter will begin to explore the effects of manipulating the testis environment in the fetal and neonatal marmoset, in addition to the effects of these treatments on human and marmoset perinatal xenografts.

6 Effects of manipulation of the gonadal environment on perinatal human and marmoset testis development

6.1. Introduction

6.1.1. *In vivo* models of perinatal germ cell development

Having established *in vivo* models of perinatal germ cell development in both the marmoset (chapters 4+5) and human (chapter 5), these models were subsequently used to investigate the effects of various manipulations of the gonadal environment on testicular development.

6.1.2. The effects of GnRHa treatment on testis development in the perinatal marmoset

The neonatal period is an important time for germ cell development in both the human and marmoset. During this period the remaining gonocytes are differentiating into spermatogonia (Rajpert-De Meyts et al., 2004) and there is a relatively high rate of germ cell proliferation (section 4.4.4.2). The neonatal period is also a period of reproductive hormonal activity (neonatal testosterone rise or 'mini-puberty') (Mann and Fraser, 1996). During this period the levels of gonadotrophins and testosterone are elevated (Kelnar et al., 2002). It has been shown that suppression of this testosterone 'surge' can be achieved in primates including the marmoset with the use of GnRHa (Lunn et al., 1994);(Marshall et al., 2005). Neonatal GnRHa treatment results in a significant reduction in testis weight as well as a reduction in germ cell number and a significant reduction in the numbers of prespermatogonia in the testes of treated males compared with their control co-twins (Sharpe et al., 2003a). It is unknown whether GnRHa can affect germ cell differentiation and proliferation during this period and in particular whether this treatment can result in a delay in maturation of germ cells, which may be relevant to the pathogenesis of TGCT (Rajpert-De Meyts, 2006).

6.1.3. The effects of phthalates on testis development in the perinatal marmoset

In-utero exposure of fetal rats to DBP administered to pregnant dams has been shown to have deleterious effects on testis development in the rat (Fisher et al., 2003). Indeed this model of in utero exposure has been used as a model for the human testicular dysgenesis syndrome (TDS) because it results in three of the four proposed features of TDS, namely cryptorchidism, hypospadias and infertility. However this model does not result in testicular CIS or TGCT, which is the other proposed disorder in TDS. The effect of phthalates on the human testis remains to be elucidated, although it has been demonstrated by epidemiological studies that certain changes in male reproductive function are correlated with levels of exposure to phthalates (Main et al., 2006). In addition, the effects phthalates on the human testis has been investigated using *in vitro* techniques. Treatment of *in vitro* cultures of human fetal testis with MEHP (a metabolite of DEHP) has been shown to result in a decrease in germ cell number, but with no effect on testosterone production (Lambrot et al., 2009). In contrast *in vitro* treatment with MBP/DBP has no effect on testosterone production by human and rat testicular explants (Lambrot et al., 2009);(Hallmark et al., 2007). This is surprising as *in vivo* treatment of neonatal marmosets with MBP results in a decrease in serum testosterone (Hallmark et al., 2007) and suggests that *in vitro* studies of the effects of phthalates may not be an adequate model for the *in vivo* situation. To date there have been no studies that investigate the *in vivo* effects of phthalates on testis development in the human.

6.2. Aims of the chapter

This chapter aimed to investigate the effects of treatment with GnRHa or phthalates on testis development during the fetal and neonatal period, using the *in vivo* systems developed in chapters 4 and 5. The focus was on the effects of these treatments on germ cell proliferation and differentiation.

6.3. Materials and methods

6.3.1. Human fetal testis tissue collection

Human fetal testis tissue collection for use in xenografting is described in Section 2.4.2. Pieces of tissue from 2nd trimester (14 weeks, n=1; 18 weeks, n=1) human fetuses were used for the DBP exposure xenografting experiments. Oral DBP administration to host mice xenografted with human fetal testis is described in section 2.6.6.2. Treatment of host mice with hCG is described in section 2.6.6.1

6.3.2. Fetal and postnatal marmoset testis collection

Marmoset testes from newborn animals exposed in-utero to MBP (section 2.5.2) were obtained as described in section 2.4.1. GnRHa treatment of neonatal marmosets is described in section 2.5.1

6.3.3. Xenografting

Castration and xenografting of testis tissue into nude mice was performed as detailed in section 2.6.1 – 2.6.5.

6.3.4. Haematoxylin and eosin staining

The method for haematoxylin and eosin staining is described in section 2.9.

6.3.5. Immunohistochemistry

Single immunohistochemistry was performed with DAB detection as described in section 2.10.2.1 - 2.10.2.6. The primary antibodies used for these experiments are listed in Table 2.1. Double staining was performed with DAB and fast blue detection as outlined in section 2.10.3. Details of conditions for double staining experiments can be found in Table 2.2.

6.3.6. Immunofluorescence

Double immunofluorescence was performed as described in section 2.11.1. The primary and secondary antibodies, and detection labels used for these experiments are listed in Table 2.3.

6.3.7. Serum testosterone radioimmunoassay

Serum testosterone levels were measured in host mice as described in section 2.7.

6.3.8. Quantification of gonocyte proportions and germ cell proliferation index

The proportion of germ cells expressing gonocyte markers in postnatal marmosets treated with GnRHa was calculated after double immunofluorescence for VASA (germ cells) and NANOG (gonocytes). The total number of NANOG positive cells was divided by the VASA positive cells. The procedure for this quantification is described in section 2.12.2.1.

The proliferation index for postnatal marmosets treated with GnRHa was calculated following double immunohistochemistry for VASA and Ki67. The quantification procedure is described in section 2.12.1 with 40 random fields counted for each section.

6.4. Results

6.4.1. The effects of gonadotrophin suppression on germ cell development in the neonatal marmoset testis

6.4.1.1. The effects of gonadotrophin suppression on germ cell differentiation in the neonatal marmoset testis

To assess the effects of neonatal GnRHa treatment on germ cell differentiation, expression of NANOG was investigated at 6 weeks of age and compared with that

of co-twin controls. NANOG and VASA co-staining allowed calculation of the proportion of the total germ cells that still expressed the gonocyte marker NANOG. The proportion of germ cells expressing NANOG was less than 1% in both control and GnRHa treated animals and there was no difference between the two groups (Figure 6.1). No NANOG positive cells were identified in three of the control testes, compared to one of the treated animals. Overall, an average of 1 in 742 germ cells expressed NANOG in the controls, compared to 1 in 163 for treated animals, however this was not a statistically significant difference.

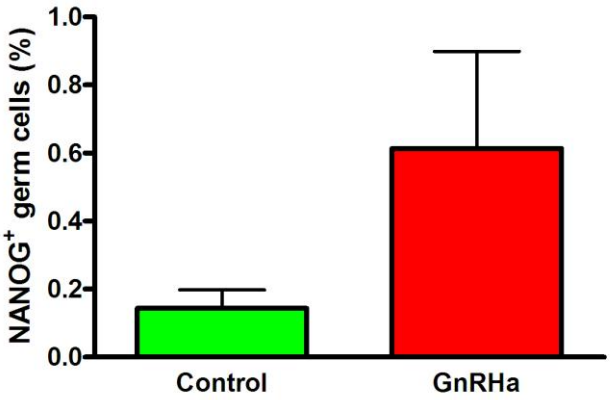

Figure 6.1. Effects of GnRHa treatment on differentiation of gonocytes in neonatal marmosets. NANOG expression in germ cells of 6 week old marmosets treated for 4 weeks with a GnRHa and comparison with co-twin controls, n=8. Paired t-test, mean ± sem. p>0.05.

6.4.1.2. The effects of gonadotrophin suppression on germ cell proliferation in the neonatal marmoset testis

The hypothalamo-pituitary-gonadal axis is active during the neonatal period in both the marmoset and human. We investigated the effects of suppressing gonadotrophins and hence testosterone production in the neonatal period, on germ cell proliferation. Treatment with GnRHa for the first four weeks of life resulted in a 30% reduction in germ cell proliferation, when compared to control (Figure 6.2).

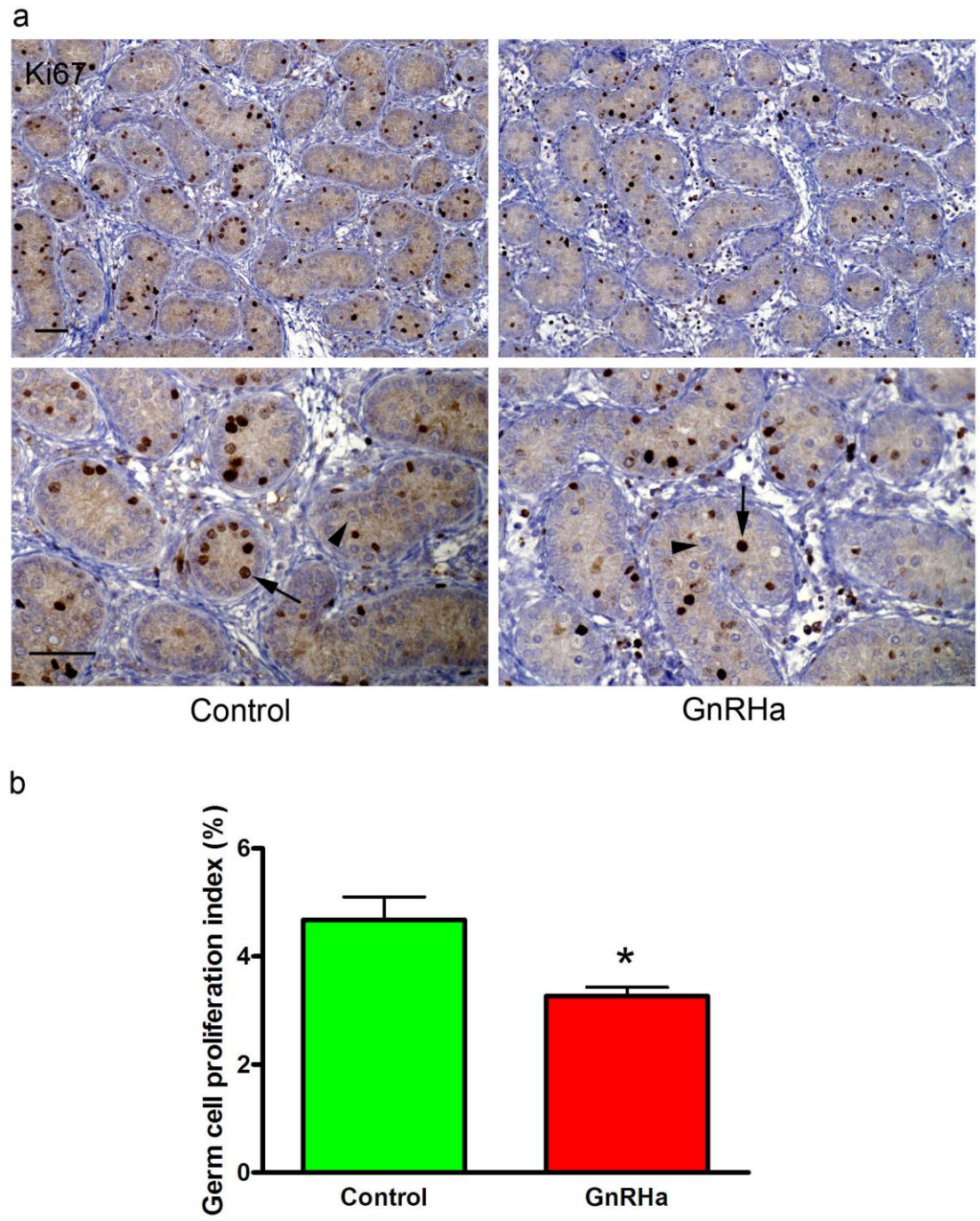

Figure 6.2. Effects of GnRHa treatment on germ cell proliferation in neonatal marmosets. a) Ki67 expression in 6 week old GnRHa treated marmosets and comparison with vehicle treated controls, n=4. Scale bars = 50 μm. Note positive (arrows) and negative (arrowhead) germ cells. Original magnification x20 (upper panels), x40 (lower panels). b) Germ cell proliferation index in 6 week old marmosets treated for 4 weeks with GnRHa and comparison to co-twin controls n=4. Paired t-test, mean ± sem. * $p<0.05$.

6.4.2. The effects of phthalates on perinatal testis development

6.4.2.1. Effects of in-utero exposure to MBP on germ cell differentiation in the marmoset during the neonatal period

Newborn marmosets exposed in-utero to MBP did not demonstrate any evidence of malformation of the reproductive system. In addition, testis weight was not different when compared to controls (Fig. 6.3).

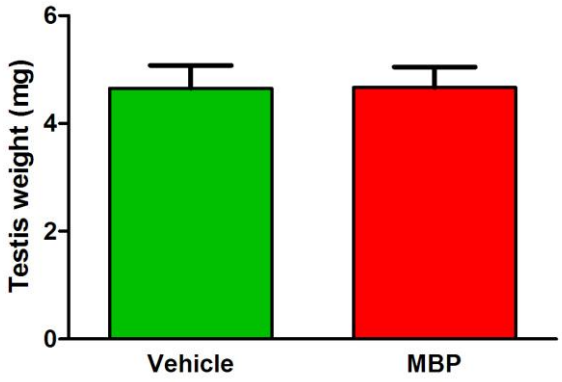

Figure 6.3. Effects of in-utero exposure to MBP on neonatal testis weight in marmosets. Testis weight for in-utero MBP treated neonatal marmosets and comparison to vehicle treated controls. n=6 MBP, n=3 controls. Unpaired t-test, mean ± sem, $p>0.05$.

In terms of expression of proteins associated with androgen action, expression of 3β-HSD and androgen receptor was detected in both treated animals and controls (Figure 6.4). 3β-HSD expression was immunolocalised to a larger proportion of Leydig cells in control compared to MBP-exposed animals. Although germ cell numbers were not affected (McKinnell et al., 2009), there was an unusual distribution of germ cells within the testes of some of the MBP-exposed animals. Large aggregates of germ cells (>10 cells/cluster) were noted within these testes when Sertoli cell cytoplasm was stained with AMH; germ cell clusters appear as unstained groups of cells (Figure 6.4).

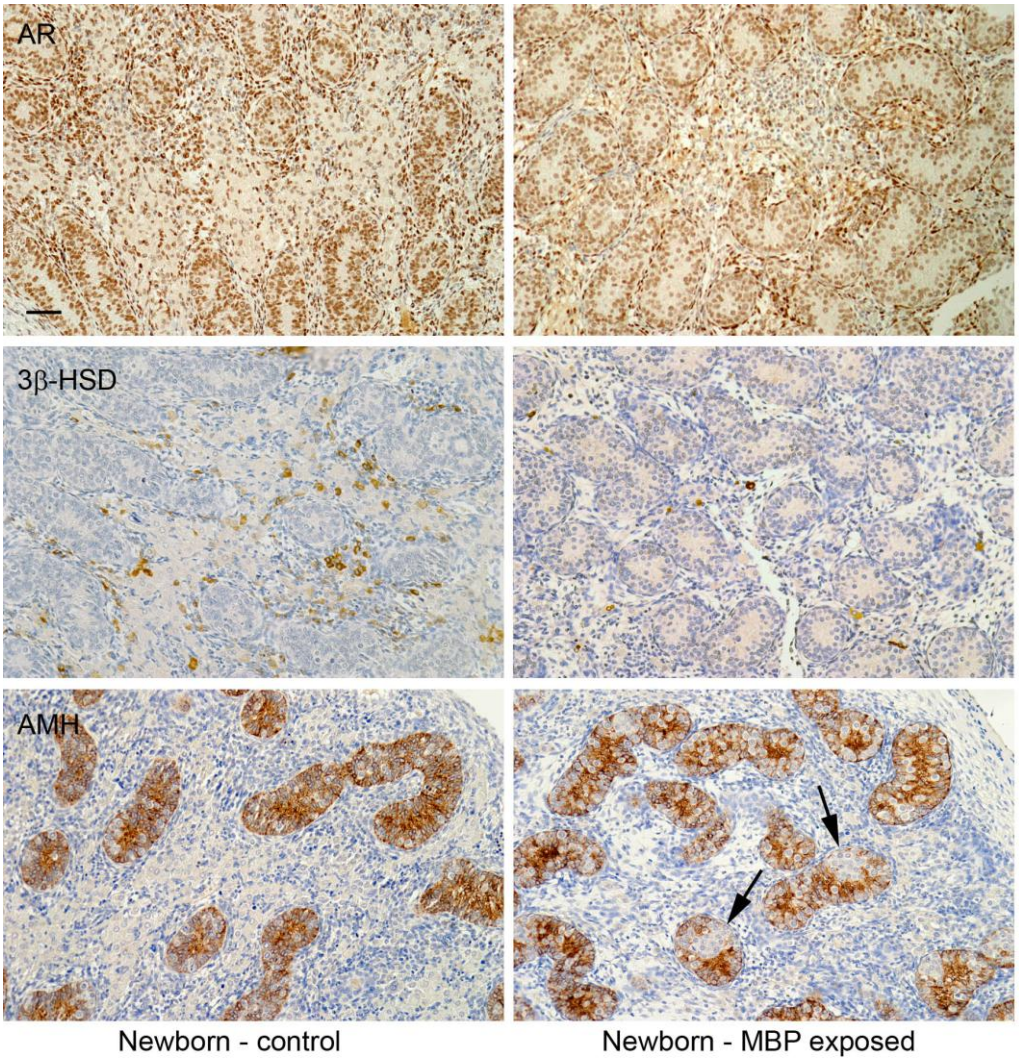

Figure 6.4. Expression of AR, 3β-HSD and AMH in newborn marmosets exposed in-utero to MBP. MBP exposed animals are compared with vehicle treated controls. All three proteins are expressed in both groups, however AMH staining revealed clusters of unstained germ cells (arrows) in some MBP-exposed animals. Scale bar = 50μm.

Further investigation of these clusters with immunohistochemistry confirmed that they were germ cells and these cells expressed OCT4 and AP-2γ, which suggests that they resembled gonocytes (Figure 6.5). In addition some of these cells were also immunopositive for VASA, when OCT4 and VASA co-localisation was performed, with evidence of the intermediate population of $OCT4^{+}/VASA^{+}$ germ cells and

OCT4^{-}/VASA^{+} prespermatogonia (Figure 6.6). In the rest of these testes expression of OCT4, AP2-γ, VASA and MAGE-A4 was similar to controls (Figure 6.5).

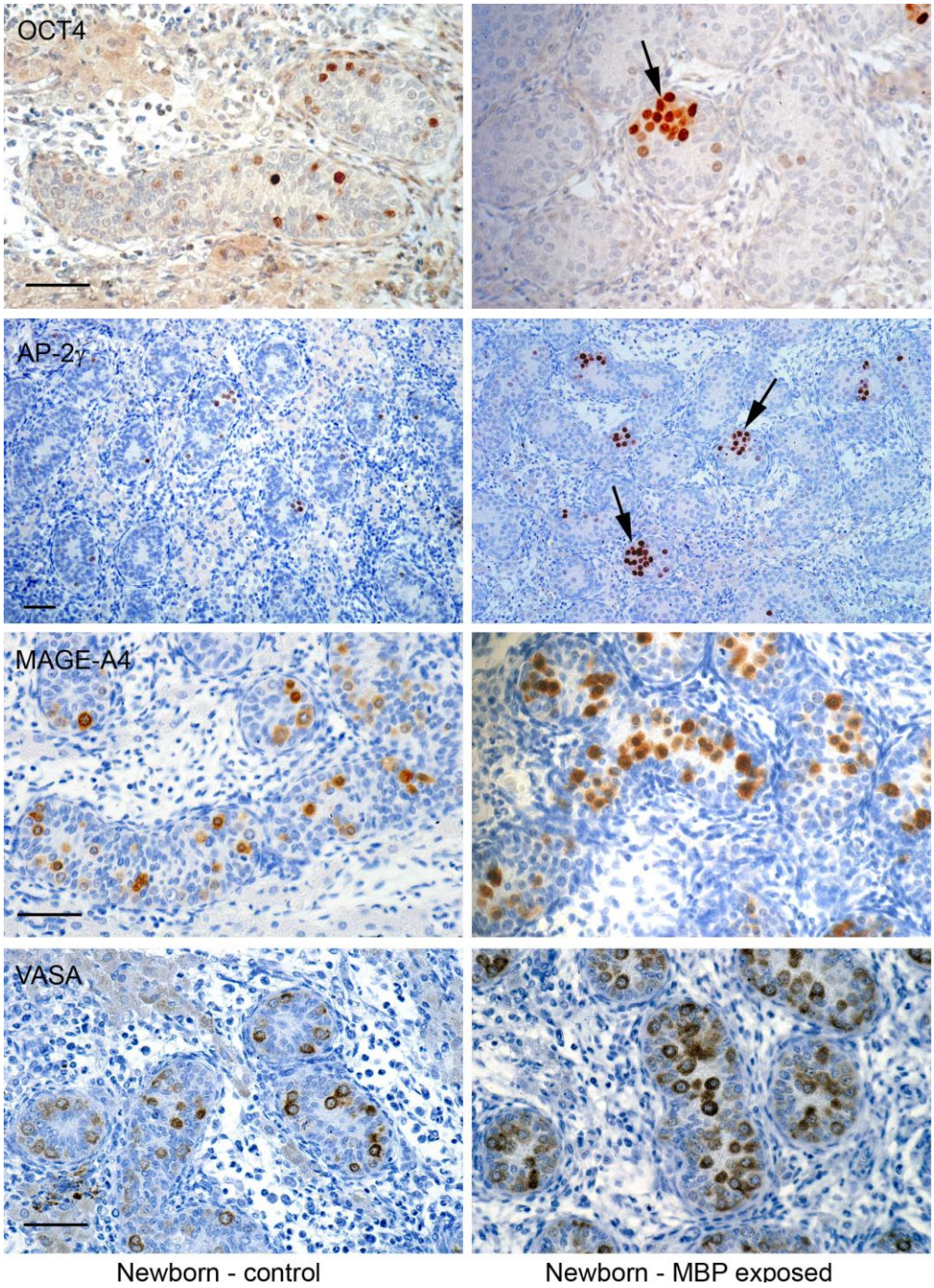

Figure 6.5. Expression of OCT4, AP2-γ, VASA and MAGE-A4 in MBP exposed newborn marmosets and controls. Germ cell clusters express OCT4 and AP2-γ (arrows), whilst VASA and MAGE-A4 are not expressed in clusters but appear uniformly distributed throughout the exposed testis. Original magnification x40 (AP-2γ x20). Scale bars = 50μm.

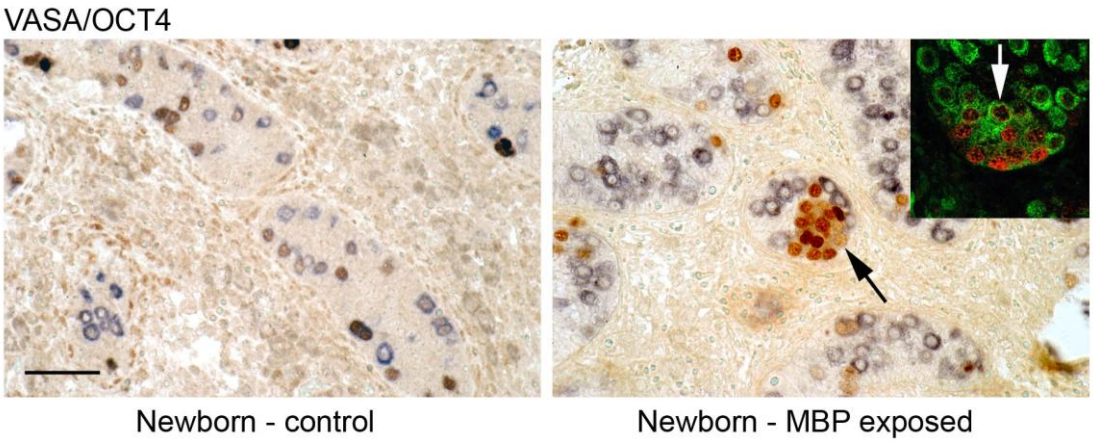

Figure 6.6. Co-expression of OCT4 and VASA in MBP exposed newborn marmosets and controls. Germ cell clusters express OCT4 (brown, black arrow), but are largely negative for VASA (blue), whilst fluorescent staining revealed that some of these OCT4 (red) positive cells also expressed VASA (inset, white arrow). Original magnification x20 (inset magnification x63). Scale bar = 50μm.

Germ cell clusters were further investigated for expression of KIT (Figure 6.7). KIT was expressed in the germ cell clusters and was demonstrated to occur in the same clusters that stained for OCT4. These $KIT^{+}/OCT4^{+}$ cells remained negative for MAGE-A4.

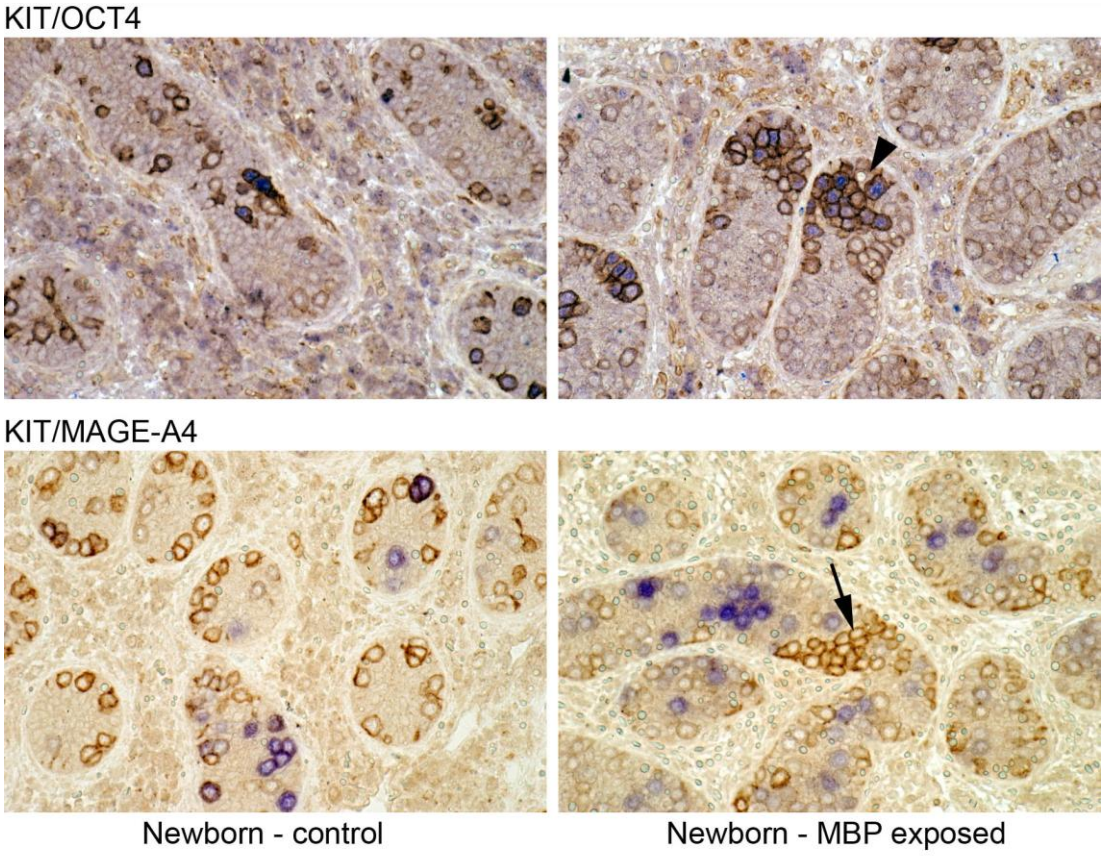

Figure 6.7. Co-expression of KIT with OCT4 or MAGE-A4 in MBP exposed newborn marmosets and controls. Germ cell clusters co-express KIT (brown) and OCT4 (blue, upper panels), whilst KIT positive clusters (arrow) do not express MAGE-A4 (blue, lower panels). Scale bar = 50μm.

The appearance of these germ cell clusters led us to speculate that there may be a process of abnormal differentiation of the germ cells within these clusters and that these may persist in older animals. Such an effect on germ cell differentiation could predispose to CIS in the developing testis. In order to test this hypothesis some of the animals exposed in-utero to MBP were allowed to live into adulthood. However, examination of the adult testis failed to demonstrate persistence of either germ cell clusters or undifferentiated germ cells (McKinnell et al., 2009).

Proliferation of cells within the seminiferous tubules of MBP exposed marmosets was also investigated by immunostaining for Ki67. Germ cell proliferation was evident in both the MBP exposed and vehicle treated controls and there was no clear difference in Ki67 expression (Figure 6.8), although quantification studies were not undertaken. It was also noted that the Sertoli cells were proliferating in both MBP-exposed and control animals.

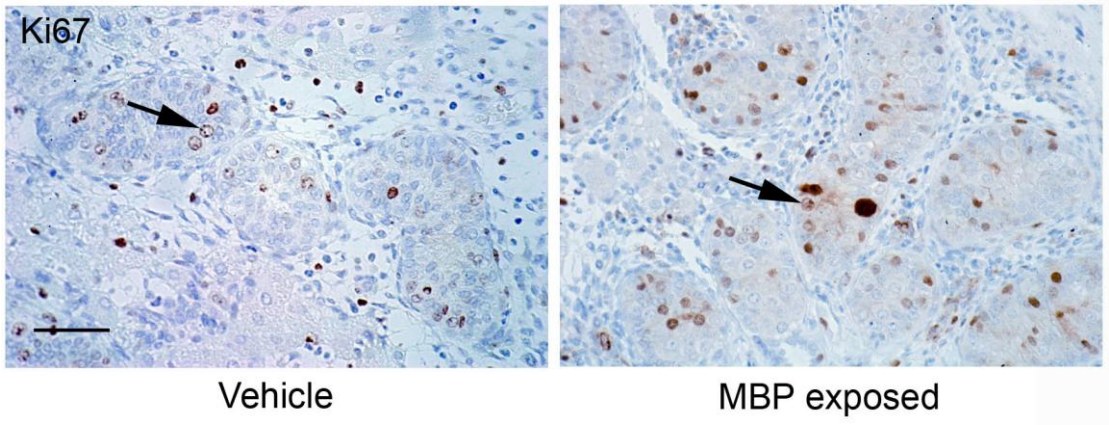

Figure 6.8. Ki67 expression in neonatal marmosets exposed in-utero to MBP. Germ cells expressing Ki67 (arrows) are seen in both the treated animals and the vehicle treated controls. Sertoli cell proliferation is also present in both sections. Scale bar = 50μm.

6.4.2.2. Effect of 6 hours of DBP treatment on human fetal testis xenograft development

Having established the xenografting approach as a good model of human fetal testis development investigation of the effect of treatment with phthalate on their development was performed. Host animals carrying second trimester (14 weeks) human fetal testis grafts were exposed to DBP for 6 hours (DBP-6h). Pieces of testis tissue from a single second trimester fetus were grafted into 6 mice (Table 6.1). The serum testosterone and seminal vesicle weights appeared to be related to whether the host animal received hCG rather than whether it received DBP 6h earlier. It was not possible to perform statistical analysis of serum testosterone or seminal vesicle

weights due to the low numbers of host animals. Although the grafts from the DBP-6h treated animals were smaller than those from vehicle treated animals this was not statistically significant (Figure 6.9).

Number of hosts	hCG	DBP	Serum testosterone (ng/ml)	Seminal vesicle weight (mg)	Grafts retrieved number (%)	Graft weights (mg)
2	✓	x	0.46	48	9 (75)	2.24
2	x	x	<0.1	18	8 (67)	1.69
1	✓	✓	1.07	63	4 (67)	1.78
1	x	✓	0.13	18	4 (67)	1.45

Table 6.1. DBP-6h treatment of host mice carrying second trimester (15 weeks) human fetal testis xenografts.

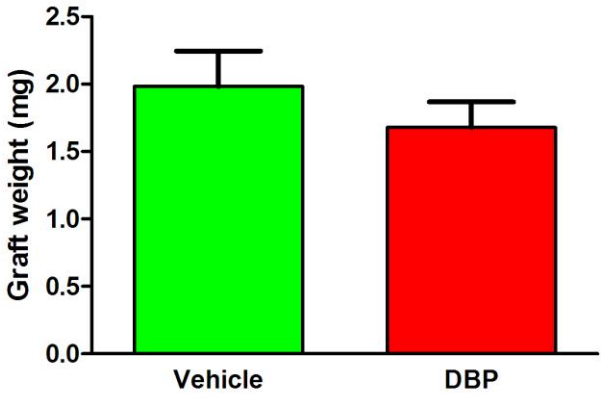

Figure 6.9. Effect of DBP-6h on second trimester graft weight. Graft weights from a single second trimester (15 weeks) human fetal testis. Grafts from host mice treated with vehicle or DBP-6h. Vehicle grafts (n=17), DBP-6h grafts (n=8). Unpaired t-test, mean ± sem, $p>0.05$.

There was no difference in morphology of the DBP-6h exposed grafts when compared to the vehicle exposed controls. Grafts from animals given hCG treatment expressed 3β-HSD and AR regardless of whether they had been exposed to DBP-6h or vehicle, although expression appeared less intense in the DBP treated grafts (Figure 6.10). There was no clear difference in germ cell differentiation

status between DBP-6h and vehicle exposed grafts, as evidenced by similar OCT4/VASA co-staining (Figure 6.11). OCT4 positive cells remained in both the DBP exposed and vehicle grafts and there was no observable difference between VASA expression between the DBP exposed and vehicle grafts.

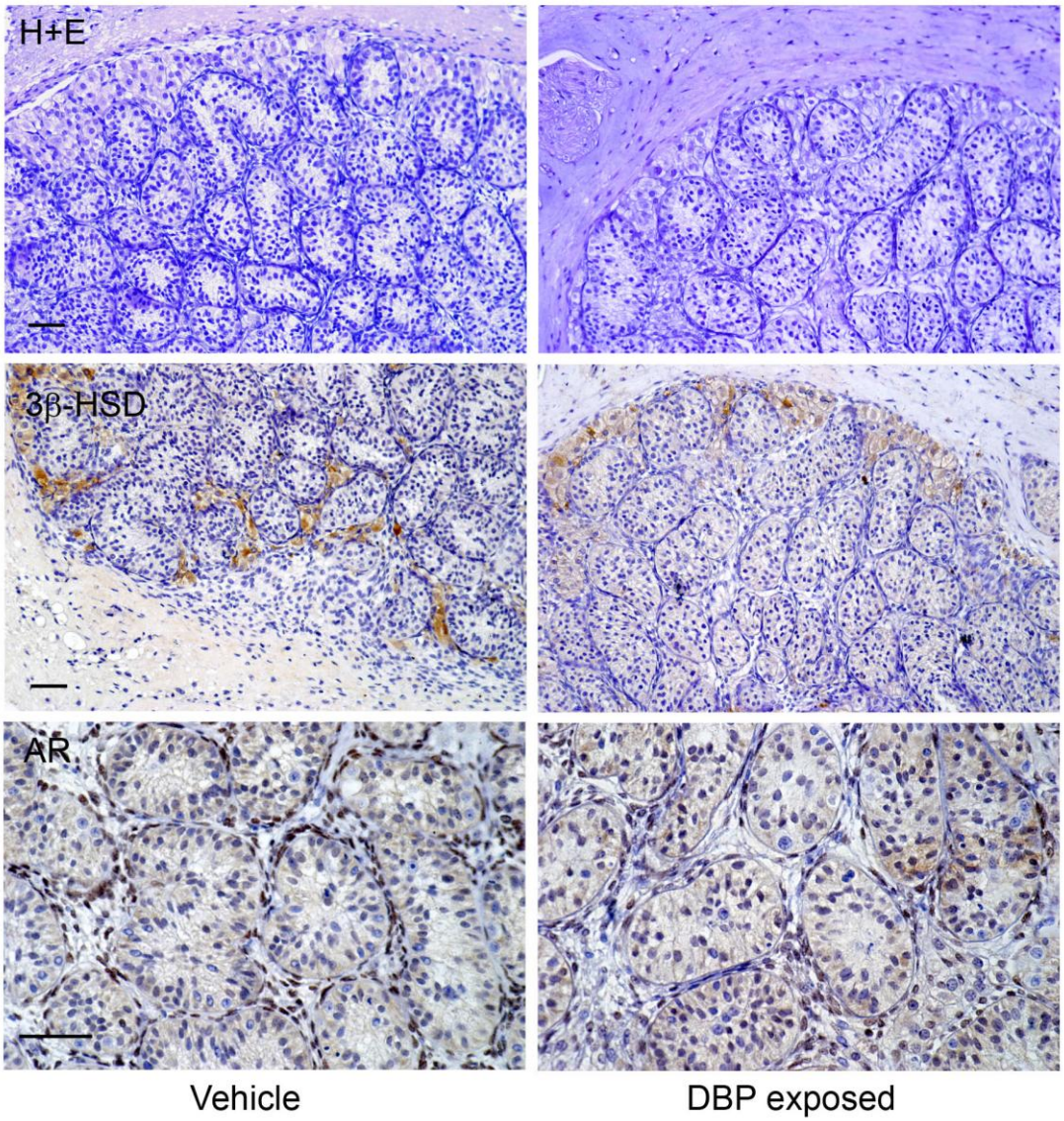

Figure 6.10. Effect of DBP-6h on tubule structure, 3β-HSD and AR expression in second trimester human fetal testis xenografts. Comparison of DBP-6h and vehicle exposed second trimester (14 weeks) human fetal testis xenografts. Host animals also received hCG. H+E staining (upper panels) demonstrates similar morphology. 3β-HSD and AR expression is comparable between DBP-6h and vehicle exposed controls. Scale bar = 50μm. Original magnification x20 (AR magnification x40).

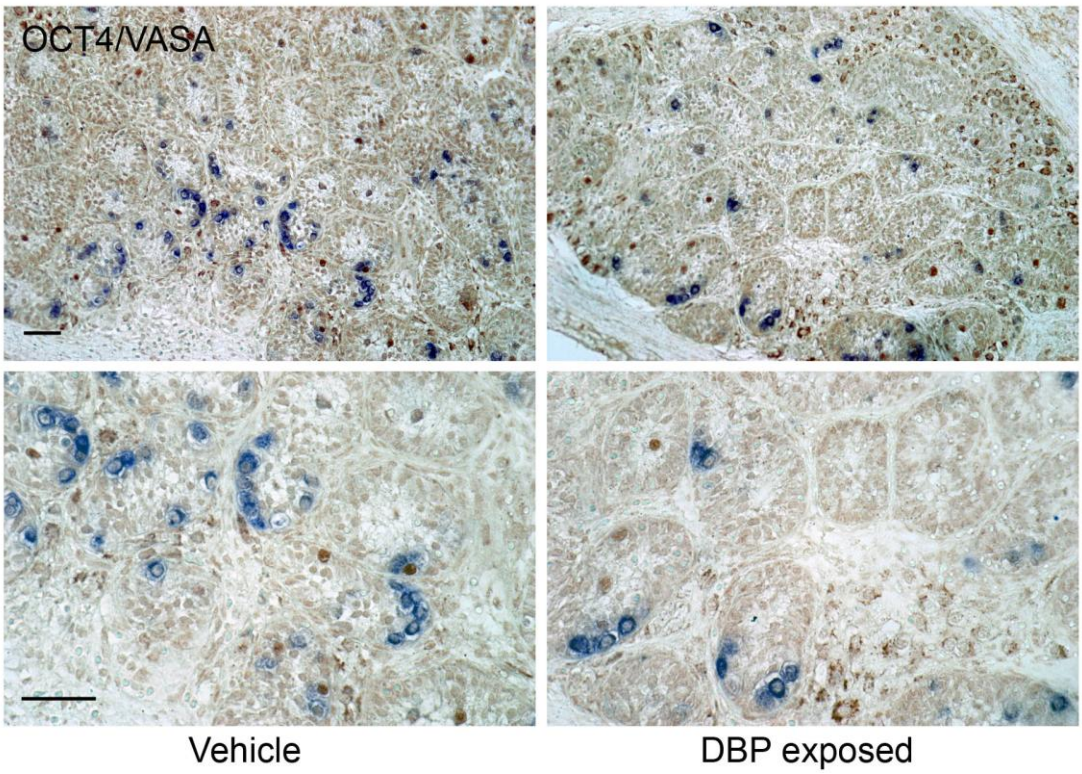

Figure 6.11. Effect of DBP-6h on germ cell differentiation in second trimester human fetal testis xenografts. Comparison of DBP-6h and vehicle exposed second trimester (14 weeks) human fetal testis xenografts. Host animals also received hCG. OCT4 (brown) and VASA (blue) are expressed in similar proportions in both DBP exposed and vehicle exposed grafts. Scale bar = 50μm. Original magnification x20 (Lower panels x40).

Proliferation of cells within the seminiferous tubules was also investigated by immunostaining for Ki67. Germ cell proliferation was evident in a small proportion of both the DBP-6h exposed animals in addition to the vehicle exposed controls (Figure 6.12). There was no clear difference between Ki67 expression in the treated animals compared to the controls and quantification studies were not undertaken. It was also noted that the Sertoli cells were proliferating in both treated and control animals.

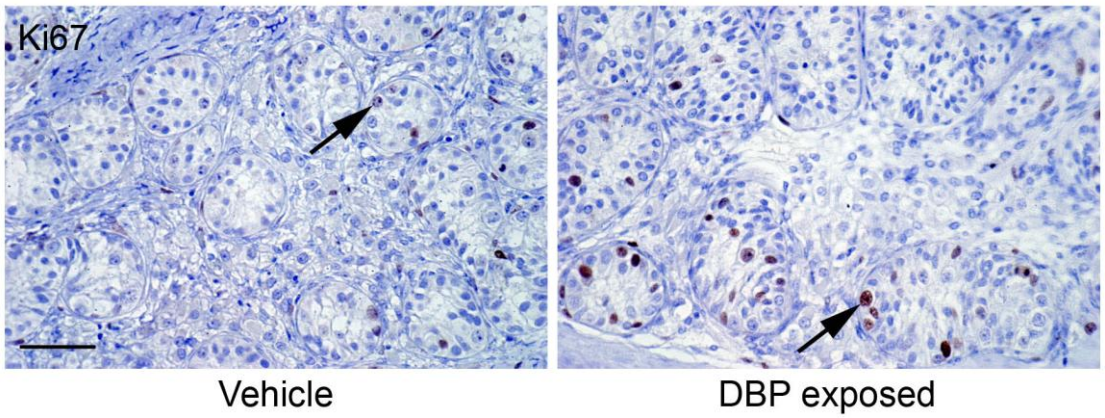

Figure 6.12. Ki67 expression in DBP-6h exposed second trimester (14 weeks) human fetal testis xenografts. Germ cells expressing Ki67 (arrows) are seen in grafts from both DBP and vehicle exposed hosts. Sertoli cell proliferation is also present in both sections. Scale bar = 50μm.

Given that there was no clear effect of DBP-6h on human fetal testis and germ cell development, host mice carrying second trimester (18 weeks) human fetal testis xenografts were treated for 78 hours with DBP (DBP-78h). All mice received hCG. Serum testosterone and seminal vesicle weights were lower in castrate hosts than in intact hosts. When castrate and intact animals were analysed separately there was a higher serum testosterone in DBP-78h treated animals than in the vehicle controls (Table 6.2). The proportion of grafts retrieved was similar for vehicle and DBP-78h overall (75% v 78%).

Number of hosts	Castrate	DBP	Serum testosterone (ng/ml)	Seminal vesicle weight (mg)	Grafts retrieved number (%)	Graft weights (mg)
1	✓	x	0.3	65	3(50)	2.73
1	x	x	9.5	485	6(100)	3.56
1	✓	✓	1.0	89	5(83)	2.40
2	x	✓	32.4	362	9(75)	3.17

Table 6.2. DBP-78h treatment of host mice carrying second trimester (18 weeks) human fetal testis xenografts.

Graft weights were not significantly different between the four groups and this was also the case when all the grafts from vehicle treated animals were compared with DBP-78h treated animals, although DBP-78h treated grafts were slightly smaller on average (Fig. 6.13).

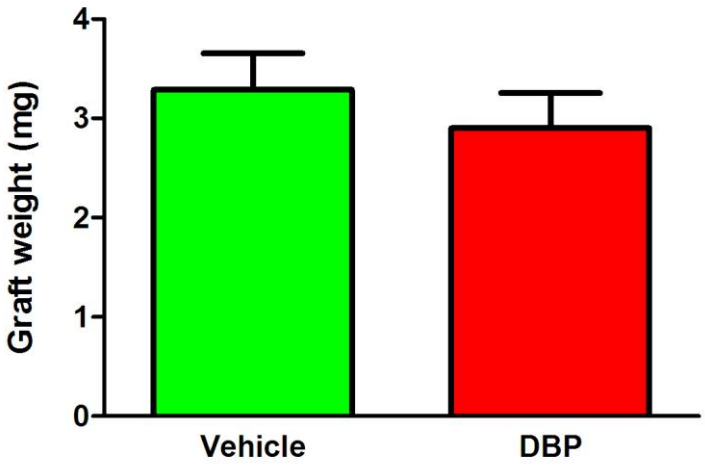

Figure 6.13. Graft weights from second trimester human fetal testis grafts exposed to vehicle or DBP-78h. Grafts were taken from an 18 week human fetal testis. All host animals received hCG treatment. Vehicle grafts (n=9), DBP-78h grafts (n=4). Unpaired t-test, mean ± sem, $p>0.05$.

Seminiferous cord structure was maintained in the DBP-78h grafts, with no obvious difference observed when compared to controls (Figure 6.14). There was also no difference between the grafts taken from intact or castrate hosts.

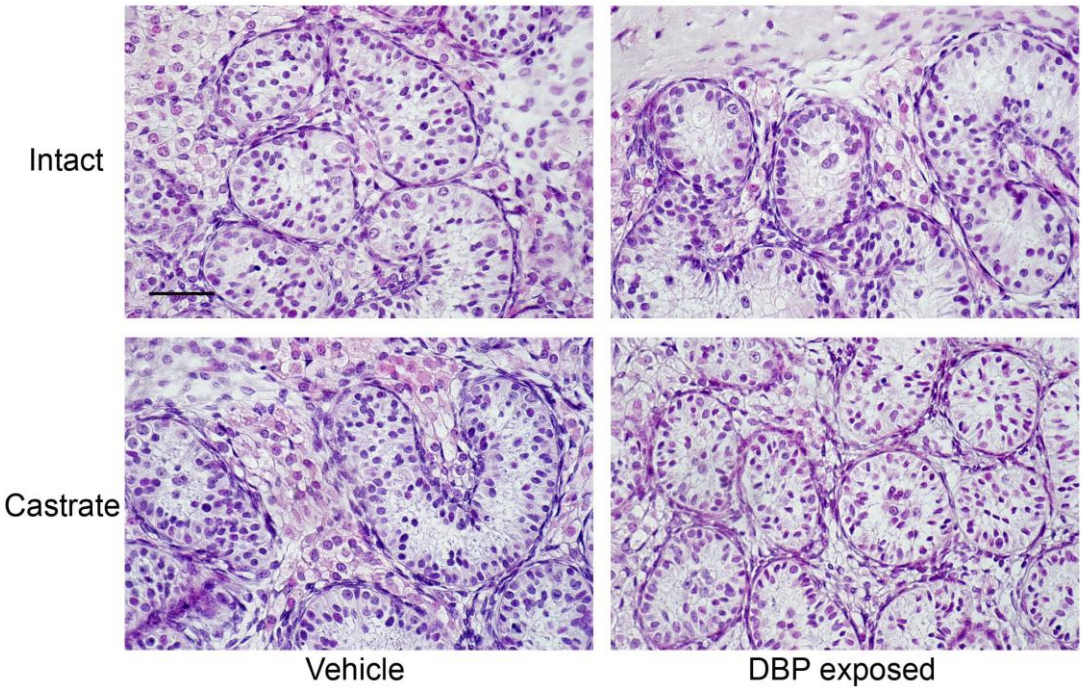

Figure 6.14. H+E staining of second trimester human fetal testis xenografts from intact and castrate hosts exposed to vehicle or DBP-78h. Grafts were taken from an 18 week human fetal testis and DBP exposed grafts are compared to vehicle exposed controls. Scale bar = 50μm.

Two of the DBP-78h exposed grafts were noted to contain a multinucleated gonocyte within the tubule (Figure 6.15). No multinucleated gonocytes were noted in the vehicle exposed animals.

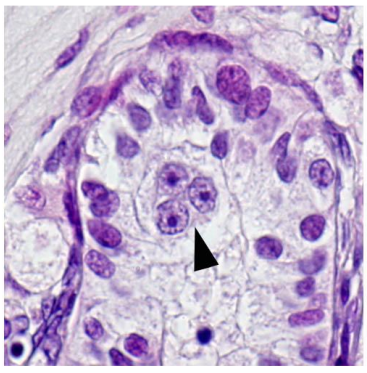

Figure 6.15. H+E. Multinuncleated gonocyte in a DBP-78h treated second trimester human fetal testis xenograft.

6.5. Discussion

6.5.1. GnRHa treatment of neonatal marmosets reduces germ cell proliferation, but does not delay differentiation of gonocytes

It is well documented that the hypothalamo-pituitary gonadal axis is more active during specific periods of life in the human and non-human primate. In the human this includes a neonatal period of high activity, during which testosterone levels approach or are within the adult range (Mann and Fraser, 1996);(Forest et al., 1973). A similar rise occurs in the marmoset and this testosterone rise can be abolished by administration of GnRHa (Lunn et al., 1994). CIS and TGCT have a significantly increased prevalence in patients with undervirilisation syndromes. In addition, these patients display delay of germ cell maturation, with persistence of germ cells expressing gonocyte markers into childhood (Cools et al., 2006b). This includes the expression of OCT4, which can be detected in the centre of the tubules in patients with dysgenetic testis beyond the first year of life (Cools et al., 2006b);(Rajpert-De Meyts et al., 2004).

It has been suggested that the neonatal testosterone rise is responsible for the ensuring that all germ cells have differentiated from gonocyte to spermatogonia by the end of infancy (Huff et al., 2001);(Hadziselimovic et al., 1986). The present studies investigated whether suppression of the neonatal testosterone rise in the neonatal marmoset testis delayed normal differentiation and proliferation of germ cells, as occurs in humans with undervirilisation syndromes (Cools et al., 2005). Expression of the pluripotency markers OCT4 and NANOG typically found in gonocytes was investigated. OCT4 is still expressed in rare germ cells at 6 weeks of age in the marmoset (Section 4.4.3.1) and also in GnRHa treated marmosets. The fact that OCT4 is still present in controls means that a large number of animals would be required to detect a significant difference in the proportion of germ cells expressing this marker. Therefore, NANOG expression was chosen for evaluation in co-twin

treated and control animals, because we have shown that most animals do not express NANOG at 6 weeks of postnatal age (Section 4.4.3.1). Less than 1% of germ cells expressed NANOG in either GnRHa treated or control testes and no significant difference was detected between the control and treated animals. It is not clear whether the trend for a slight increase in NANOG expression in some treated animals could result in persistence of occasional gonocytes beyond 6 weeks of age, although preliminary studies of animals treated neonatally with GnRHa and killed at 14 weeks of age have not demonstrated any persistence of gonocyte markers. These results indicate that suppression of gonadotrophins during the neonatal period does not delay differentiation of gonocytes. It is recognised that in patients with AIS, the risk of CIS and TGCT is much higher for those with PAIS than patients with CAIS. Indeed the malignancy risk is 50% for PAIS and only 2% for CAIS (Looijenga et al., 2007b). In addition, patients with hypogonadotrophic hypogonadism are not at increased risk of CIS/TGCT, despite having cryptorchid testes (Looijenga, 2009). These results suggest that delay in germ cell differentiation may be related to reduced, rather than a complete suppression of testosterone levels or testosterone action.

The present studies also investigated whether germ cell proliferation could be affected by GnRHa suppression of gonadotrophins and testosterone activity. It has previously been demonstrated that GnRHa treatment of the marmoset during the childhood phase of development does not significantly affect the expression of PCNA, compared to controls (Kelnar et al., 2002). In this study the expression of PCNA in germ cells was evident in just over 40% of cells for both treated and control animals. The neonatal period has been described as a period of active proliferation of germ cells (Chemes, 2001) and the present studies have shown that animals treated neonatally with GnRHa have a significantly reduced rate of germ cell proliferation, compared to the controls. However complete suppression of germ cell proliferation did not occur. The results of the present studies are in keeping

with results in previous studies that demonstrated a 27% reduction in the increase of germ cell numbers in marmosets treated with GnRHa from birth to 18-24 weeks, compared to controls (Sharpe et al., 2003a). This is similar to the 30% decrease in germ cell proliferation in animals treated from birth to 4 weeks in the present studies. These results suggest that germ cell proliferation in the neonatal marmoset testis is partially dependent on gonadotrophins and testosterone, but that other as yet unidentified factors are also important. In the human there are high rates of germ cell proliferation in the first three months of life, which coincides with the high levels of gonadotrophins and testosterone in the neonatal period (Berensztein et al., 2002). However the highest rates of proliferation are seen in the first month of life when these hormones are not at their peak. Therefore in addition to gonadotrophins, other factors that are high in the neonatal period such as growth hormone or prolactin may be important (Berensztein et al., 2002).

The reverse situation occurs in the adult primate testis, compared with the neonatal testis. In adult rhesus monkeys, germ cell differentiation in spermatogonia is absolutely gonadotrophin dependent, whilst proliferation of A_d, A_p and B spermatogonia is independent of gonadotrophins (Marshall et al., 2005). Proliferation of these spermatogonia was unchanged when animals treated with GnRHa alone were compared with animals that had received GnRHa followed by testosterone or FSH or a combination of both (Marshall et al., 2005). GnRHa resulted in a failure of differentiation of germ cells beyond type A spermatogonia in adult rhesus monkeys, whilst the replacement of testosterone +/- FSH resulted in the resumption of full spermatogenesis with an increase in the numbers of differentiating spermatogonia in the testosterone + FSH group (Marshall et al., 2005). In the present studies using marmosets, results have demonstrated that although levels of gonadotrophins and testosterone are high during neonatal and adult life, it appears that the mechanisms controlling germ cell proliferation and differentiation during these two periods are different.

6.5.2. In-utero phthalate treatment does not result in malformation of the reproductive tract or alteration of androgen levels

In the rat model of TDS, treatment of pregnant dams with 500mg/kg DBP results in high rates of reproductive tract malformations such as cryptorchidism and hypospadias in their offspring (Fisher et al., 2003);(Mylchreest et al., 1998). No such abnormalities were noted in the newborn marmosets exposed in-utero to MBP. Given the high percentage of DBP-exposed rats that exhibit these abnormalities it would be expected that if these effects also occurred in marmosets then of the 11 marmosets that were exposed to MBP, up to 7 of them would have cryptorchidism and 2 would have hypospadias (McKinnell et al., 2009). The failure to find any of these abnormalities does not rule out the fact that MBP may have an effect, but it would appear that the marmoset fetus is relatively resistant to perturbations as a result of exposure to MBP as compared with the rat. In rats, exposure to DBP has an impact on androgen dependent tissues and the levels of intratesticular testosterone have been shown to be reduced by 70-90% in the fetus towards the end of in-utero treatment (Fisher et al., 2003); treatment with 1000mg/kg MBP has been shown to reduce fetal intratesticular testosterone levels by >90% (Shono et al., 2000). However, postnatal serum testosterone levels in DBP exposed rats were not significantly different from those of controls at 4 days of postnatal age, despite the suppression that had occurred during fetal life (Fisher et al., 2003). The neonatal marmosets exposed in-utero to MBP did not have significantly different serum testosterone levels to control animals at birth, although it is not known whether testosterone had been affected during the treatment period. However it is notable that direct administration of MBP to neonatal marmosets results in a significant reduction in serum testosterone levels 5 hours after treatment (Hallmark et al., 2007) and it is possible that this is the case for the in-utero treated marmosets, however this remains to be determined.

DBP (500mg/kg/day) treatment of host mice carrying second trimester human testis xenografts did not reduce serum testosterone production after 6 or 78 hours when compared to vehicle treated controls and this was regardless of treatment with hCG. In fact testosterone levels and seminal vesicle weights were highest in the host animals that received DBP. This would appear to be at odds with the results reported by Swan *et al* who demonstrated an inverse relationship between the anogenital index (AGI, a reflection of fetal testosterone levels) of children and the prenatal maternal urinary MBP level of the mothers (Swan et al., 2005). However another study failed to show an association between maternal MBP levels and AGD in male offspring (Huang et al., 2009). In the current study we also demonstrated that the testes from MBP treated neonatal marmosets and DBP exposed human fetal testis xenografts expressed both AR and 3β-HSD, indicating that the tissue maintained a capacity for steroid biosynthesis as well as the potential to respond to testosterone. Expression of 3β-HSD appeared to be in relatively fewer cells in DBP exposed human fetal testis xenografts than in vehicle exposed grafts, but this was not quantified. Based on our observations it appears that the effects of phthalates on testosterone production by marmoset and human testis are not as pronounced as in the rat, suggests that phthalates may have a less striking impact on developmental competence of the reproductive tract than that inferred from rodent studies

6.5.3. Phthalate treatment may have testosterone independent effects on testis development

6.5.3.1. In-utero MBP causes increased undifferentiated germ cell clustering in neonatal marmosets

In some, but not all, newborn marmosets exposed in-utero to MBP there were differences in germ cell distribution with large clusters of germ cells located in regions throughout the testis, although quantification of germ cells revealed no difference in overall germ cell number (McKinnell et al., 2009). This contrasts with the situation in the rat in which a reduction in germ cell number is seen in DBP

exposed animals (Ferrara et al., 2006). Occasional germ cell clusters occurred in the control animals but they were found less frequently, in fewer animals and each cluster contained fewer germ cells. The cells within the germ cell clusters in MBP-exposed newborns displayed the phenotype of gonocytes as the expressed proteins such as OCT4, NANOG, AP2γ and KIT. This included co-expression of KIT and OCT4, which has been shown to be characteristic of gonocytes (Honecker et al., 2004);(Gaskell et al., 2004). This was further confirmed by the lack of expression of MAGE-A4 in these KIT expressing clusters (Figure 4.12). These expression profiles are in keeping with normal differentiation of gonocytes ($OCT4^{+}/KIT^{+}/MAGE\text{-}A4^{-}$) via intermediate germ cells ($OCT4^{low/-}/KIT^{-}/MAGE\text{-}A4^{-}$) to prespermatogonia ($OCT4^{-}/KIT^{-}/MAGE\text{-}A4^{+}$) (Gaskell et al., 2004). No atypical germ cell expression profiles were identified in the clusters. In the MBP-exposed animals, the spermatogonia expressing VASA or MAGE-A4 appeared to be distributed throughout the tubules in a similar manner to controls, although germ cells in transition ($OCT4^{+}/VASA^{+}$) were occasionally noted within the clusters. Prolonged expression of OCT4 in germ cells has been demonstrated in the DBP exposed fetal rat (Ferrara et al., 2006) and the appearance of these gonocyte clusters led us to investigate whether cells expressing gonocyte markers persisted into adulthood. There was no difference between the relative numbers of cells with the different phenotypes and when adult animals that had been exposed to MBP in-utero were analysed there were no germ cell clusters and importantly no germ cells were identified that expressed OCT4, NANOG or AP2γ (McKinnell et al., 2009). This rules out the persistence of undifferentiated fetal germ cells and this suggests that in-utero MBP treatment using the current regimen does not induce CIS in the marmoset.

Despite the finding of germ cell clustering in the MBP exposed marmosets, there were no similar effects in the DBP exposed human fetal testis xenografts. Both the distribution and relative proportion of the germ cells was similar in both treated and control grafts. This included both gonocytes and spermatogonia. Although

there was no such clustering it is important to note that the choice of phthalate and the treatment regime was different for the marmoset and human studies. We have already demonstrated in the previous chapter that the xenografting technique provides a comparable model to normal development (chapter 5) and that the development of germ cells within the testis is similar between the marmoset and human (chapter 4).

6.5.3.2. Germ cell proliferation is not affected by in-utero MBP exposure in marmosets or by DBP in human fetal testis xenografts

Previous studies have demonstrated an effect of phthalate exposure on perinatal germ cell proliferation in rats (Ferrara et al., 2006). In-utero exposure of rats to DBP resulted in delayed exit from quiescence in germ cells between postnatal day 4-8, with a 28% reduction in Ki67 expression (Ferrara et al., 2006), whilst a study of human fetal testes cultured for 3 days with MEHP did not demonstrate an effect on proliferation despite a reduction in germ cell number (Lambrot et al., 2009). We have shown that germ cell proliferation continues normally in newborn marmosets exposed in-utero to MBP and that germ cell number was not altered by treatment. Proliferation of $VASA^+$ germ cells was variable between individuals within both groups, but there was no difference between the number of proliferating germ cells in MBP treated animals and controls (McKinnell et al., 2009). Importantly it was also noted that the germ cell clusters did not contain an abnormally high expression of Ki67 positive cells (McKinnell et al., 2009). In human fetal testis xenografts we observed proliferation of germ cells in grafts from both vehicle and DBP treated hosts with no clear difference between the two groups. Overall these results indicate that phthalate treatment as assessed using the methods described does not have any striking effect on germ cell proliferation.

6.5.3.3. DBP may reduce human fetal testis xenograft weight

Despite the lack of evidence that there is an effect of phthalates on androgen production in marmoset and human testes, we have demonstrated effects of these

chemicals on other aspects of testis development. Testosterone independent effects on germ cells have also been demonstrated in the mouse, a species in which testosterone production is not affected by phthalates (Gaido et al., 2007). Mice treated with DBP exhibit an increased number of multinucleated gonocytes per cord compared with controls, without a measurable decrease in testicular testosterone. Similarly, *in vitro* studies of fetal human testis showed that four days of MEHP treatment resulted in germ cell apoptosis without affecting testosterone production in the explants (Lambrot et al., 2009). Testis weight at birth has been shown to be significantly decreased in rats exposed to DBP (Fisher et al., 2003). In our studies testis weight in neonatal marmosets treated in-utero with MBP was not significantly different to controls. Human fetal testis grafts from hosts treated with DBP were smaller by 25% (DBP-6h) and 12% (DBP-78h), than the corresponding vehicle-exposed grafts although this was not statistically significant. It is possible that the low numbers of human fetal testis grafts and host animals may mask a significant effect on graft weight and therefore the numbers should be increased. However the 25% decrease in graft weight occurred in the grafts that had only been exposed to DBP for 6 hours and it is unlikely that such a short period of treatment would produce such an effect, so the weight changes may be incidental.

6.5.4. Phthalate treatment regimes vary between studies and this may be reflected in the variable results

When interpreting the results of phthalate treatment studies there are several factors that must be taken into account. Firstly the choice of compound is variable between studies. Many studies have used DBP, which requires metabolism into the active compound MBP. There may be differences in metabolism that could account for some of the differences between species. DBP treatment can result in a rat model of TDS. Maternal and fetal concentrations of MBP following DBP (500mg/kg) treatment in mice have been shown to be at least equivalent to those attained in the rat (Gaido et al., 2007) and it was assumed that the doses used in rats to induce TDS would result in equivalent exposure of the xenografts to the active metabolite.

However, this dose of DBP did not suppress testosterone production from the xenografts or from intact host mice. It is unknown how much DBP and MBP the graft tissue is exposed to and this may be affected by variation in metabolism of DBP in host mice, compared to rats. As a result the dose may need to be altered, particularly as the effects of DBP in the rat are dose-dependent (Mylchreest et al., 1998). In this regard it may be useful to assess the effects of DBP on xenografts using a nude rat host, which has the same immunodeficiency phenotype as nude mice (Cash et al., 1993). Whilst DBP was used for treatment of human fetal testis xenografts, MBP was used for in-utero marmoset treatment because the marmoset has been described as a poor metaboliser of diesters into their active monoester metabolites (Rhodes et al., 1986). The timing of treatment is also variable between studies. This may be particularly important because a key window of development, during which suppression of androgen levels will result in reproductive tract abnormalities has been described for the rat (Welsh et al., 2008). The present studies extrapolated from the timing of the 'masculinisation window' in fetal rats for DBP exposure in human fetal testis xenografts. However, these grafts were only exposed during a short period of fetal development and this may explain why effects on germ cells were not seen as it is still possible that treatment did not coincide with a critical window of development.

6.5.5. Conclusion

This chapter has demonstrated that suppression of testosterone with GnRHa in the neonatal marmoset does not significantly affect germ cell differentiation. This suggests that persistence of germ cells with a gonocyte phenotype beyond infancy, as occurs in the pathogenesis of TGCT is not simply a consequence of suppression of gonadotrophins and testosterone. We have also demonstrated that germ cell proliferation in the marmoset is partially gonadotrophin dependent as opposed to other periods of life such as the childhood period. Exposure of human and non-human primate testes to DBP/MBP in-utero or neonatally has failed to demonstrate

the reproductive and testicular abnormalities that are seen in the rat model of TDS, although there were some possible effects on germ cell development in the marmoset exposed in-utero to MBP. However, given the small numbers of animals treated these results should be considered preliminary. Further study is required to determine the optimum conditions for phthalate treatment including the appropriate phthalate ester, dose, timing and duration, before firm conclusions on the effects of these chemicals on human testis development can be made.

7 Final discussion

This thesis aimed to investigate the development of the human fetal and early postnatal testis, with particular emphasis on the origins and pathogenesis of TGCT. Initial investigation focused on the origins of the pre-neoplastic CIS cells by analysing sections of testis taken from adult patients with TGCT (chapter 3). It became clear that models of in vivo testis development during fetal life would potentially provide more insight into the development of CIS and TGCT. Two approaches were used to develop such in vivo models. The first approach was to investigate the Common Marmoset monkey as an animal model of human testis development during fetal and postnatal life (chapter 4), This species was selected as previous studies had determined that the organisation of spermatogenesis in adult marmosets closely resembles that in the human (Millar et al., 2000). A second approach involved the use of testicular xenografting to attempt to recapitulate normal testis development in the human and marmoset using a nude mouse host (chapter 5). Finally the thesis aimed to disrupt normal testis development in these in vivo models using hormonal suppression and endocrine disruptors that are known to affect testis development or result in testicular dysgenesis in other species (chapter 6).

CIS cells are postulated to arise from transformed fetal gonocytes, based on several factors such as morphology and expression of various protein markers (Skakkebaek et al., 1987):(Rajpert-De Meyts, 2006), and heterogeneity in expression of these proteins has been described (Rajpert-De Meyts et al., 1996). Although some of these proteins (e.g. MAGE-A4) are described as being present in a subpopulation of CIS cells, others (e.g. OCT4, VASA) are described as being present in all CIS cells (Rajpert-De Meyts, 2006), (Looijenga et al., 2003a). The present studies have characterised the protein expression profiles of the subpopulations of CIS cells using double and triple immunohistochemical techniques. This approach has revealed that expression of proteins previously described as being present in the majority of

CIS cells, such as MAGE-A4 and VASA are only expressed in a small proportion of the CIS cell population. For example MAGE-A4 and VASA were only present in a few CIS cells, whilst most of the germ cells within a CIS-containing tubule that express MAGE-A4 and VASA are not CIS cells. Classification of CIS cells is based on morphological appearance and expression of proteins such as OCT4, which has been reported to be present in all CIS cells (Looijenga et al., 2003a). The present study has demonstrated that there is a population of CIS cells that do not express OCT4 and this is based on the finding that some of the OCT4 negative germ cells express other markers of CIS, such as PLAP. It could be argued that the OCT4 negative CIS cells may in fact be cells that have progressed to invasive disease, however these OCT4 negative cells are also present in pre-pubertal patients prior to the formation of a tumour, which suggests that they are CIS cells. It remains to be determined what the relevance of the different CIS phenotypes is to the subsequent development of TGCT. However, there are implications for studies that rely on accurately identifying CIS cells, such as recent studies that use laser microdissection of CIS cells to analyse the gene expression profiles of these cells (Sonne et al., 2009). The quality of the data gained in these studies depends on being able to accurately discriminate between CIS and non-CIS cells. However as the present studies have shown that the use of expression of a single 'CIS' marker will not identify all CIS cells and conversely the lack of such protein expression does not rule out the possibility that a cell is a CIS cell validation and interpretation of data generated in studies where cell identity is important will need to take these findings into account.

The present studies have also identified subpopulations of germ cells that have protein expression phenotypes distinct from those found in normal human fetal germ cells. Whilst the majority of the protein expression profiles in CIS are also found in fetal germ cells and support the concept of a fetal origin for CIS cells (Skakkebaek et al., 1987);(Rajpert-De Meyts, 2006), the finding of these unique expression phenotypes in CIS cells suggests that there may be downregulation of

some of these proteins in a subset of the CIS cells. No association was demonstrated between the individual subpopulations and the resulting tumour type and it remains to be determined what the significance of the different expression profiles might be. The present studies have also demonstrated that proliferation of germ cells is variable in the various subpopulations of CIS cells. The populations of cells expressing a gonocyte phenotype ($OCT4^+/VASA^-$ or $OCT4^+/MAGE\text{-}A4^-$) have higher proliferation rates than those that express a differentiating phenotype. The relevance of the increased proliferation in these subpopulations of CIS cells is unclear but it could be related to the invasive potential of the subpopulation. Interestingly the proliferation rate of the various subpopulations of CIS cells was very similar to that demonstrated in the normal human fetal testis and includes both *in situ* human fetal testes and xenografted testes and this suggests that CIS cells may retain the proliferation profile of human fetal testes and that the xenografting model is a suitable system in which to investigate proliferation of germ cells during fetal life.

In the normal human fetal testis, there is a mixture of germ cells with different phenotypes within a single tubule (Gaskell et al., 2004) and this is also the case for CIS cells (Rajpert-De Meyts et al., 1996). The present study has described the expression profiles in more detail and also identified expression profiles that have not been described previously. In the normal human fetal testis, differentiation of germ cells from gonocytes into spermatogonia occurs throughout fetal life and into the postnatal period and a mixture of cells at different stages of this process are found within an individual tubule at any given time. The prolonged period of gonocyte differentiation in humans may predispose to the development of CIS given the right circumstances (Rajpert-De Meyts, 2006). Persistence of gonocytes beyond the normal period of development into spermatogonia has been proposed to result in CIS and this is supported by the fact that delay in germ cell differentiation occurs in patients with DSD, a population that are at increased risk of TGCT (Cools

et al., 2006b). The present studies have demonstrated that the same pattern of prolonged and asynchronous differentiation from gonocytes to spermatogonia occurs in the normal marmoset testis during fetal and early postnatal life. In addition, the differentiation of germ cells in fetal life has been replicated in an *in vivo* xenografting model for both the human and marmoset, including the prolonged and asynchronous differentiation of gonocytes. The development of these *in vivo* systems provide a unique opportunity to test the hypothesis that CIS cells result from delayed differentiation of fetal germ cells and that asynchronous differentiation of these cells may predispose to the formation of CIS cells. Two approaches were used to attempt to induce delay in gonocyte differentiation or CIS using the marmoset and human testis. Previous studies have demonstrated that exposure of fetal rats to the phthalate DBP results in a collection of abnormalities characteristic of human TDS (Fisher et al., 2003). Notably, this does not include CIS and TGCT. If the hypothesis of asynchronous gonocyte differentiation predisposing to CIS is correct, then this may explain why CIS has not been described in the rodent and would also leave open the possibility that phthalates may induce CIS in the human and marmoset. The present studies explored exposure of fetal marmoset and human testes to the phthalate DBP (MBP) using different *in vivo* approaches. Although one exciting observation obtained during these studies was that a proportion of marmosets exposed in utero to MBP exhibited gonocyte clusters within the seminiferous cords at birth, these did not persist into adulthood and there was no evidence of cells with a CIS phenotype. In xenografts from fetal human testes exposed to DBP (via mouse hosts) there was no apparent effect on germ cell differentiation or proliferation. It remains unclear whether there are effects of phthalates on human fetal testis development or whether the lack of effect in the present studies is due to the choice of treatment, timing of exposure or duration of treatment. A programming window has been described in rats, during which masculinisation of the reproductive tract can be disrupted by anti-androgens (Welsh et al., 2008). Notably, the consequences of treatment administered only during this

critical period of development included some of those described for TDS, such as cryptorchidism and hypospadias. The timing of this programming window has been extrapolated from rat to human and is believed to be between 8-14 weeks gestation (Welsh et al., 2008). Further studies of disruption of androgen action should focus on treatment of hosts mice carrying human testicular xenografts taken from fetuses between 8-14 weeks gestation as the masculinisation programming window may have been missed in the current studies.

The second model system used in an attempt to induce germ cell maturation delay, CIS and TGCT was based on the finding that TGCT are more common in patients with undervirilisation syndromes (Cools et al., 2005). In addition, differentiation of spermatogonia has been shown to be gonadotrophin dependent in the adult rhesus monkey (Marshall et al., 2005). These results suggest that suppression of gonadotrophins and testosterone may also delay germ cell differentiation in the perinatal period. The present study investigated the effect of GnRHa treatment of marmosets in the neonatal period and did not demonstrate a delay in gonocyte differentiation. These results suggest that gonadotrophins are not responsible for the differentiation of gonocytes to spermatogonia as has been suggested (Hadziselimovic et al., 1986), (Huff et al., 2001). The factors that result in delay in germ cell maturation remain to be elucidated, however it must be noted that the treatment was given to neonatal marmosets and this timing is not within the proposed male programming window as described earlier. Therefore it would be important to establish the effects of suppressing androgens during fetal life on germ cell maturation. Interestingly, GnRHa treatment of neonatal marmosets did result in a partial decrease in germ cell proliferation, compared to vehicle treated controls. Overall, the results of GnRHa treatment of neonatal marmosets showed opposite effects of gonadotrophins on germ cell differentiation and proliferation to those found in the adult rhesus monkey. The present study found that germ cell proliferation in neonatal marmosets is partially gonadotrophin dependent and

differentiation is gonadotrophin independent, whilst a previous study in the adult rhesus monkey demonstrated that proliferation is gonadotrophin independent, whilst differentiation is gonadotrophin dependent (Marshall et al., 2005).

A major finding of the present studies was that xenografting of first trimester testis tissue could support the formation of testis cords. Seminiferous cord formation starts on or about 7 weeks of gestation and is variable between individuals (Gaskell et al., 2004). In the present studies, seminiferous cords were not fully formed at 9 weeks of gestation, although in some fetuses the process had begun. Most of the understanding of the initial stages of testis development and cord formation comes from rodent studies (Wilhelm et al., 2007) and the present studies have provided the first *in vivo* system for investigating human testicular cord formation. This system can be used to investigate normal development during this period, but importantly it can also be used to investigate disruption of the development of the testis and therefore this system could be used to investigate DSD in humans. DSDs result from failure of normal gonadal development and/or the effects of abnormal androgen production, synthesis or action. One such approach may involve the use of anti-androgens to block testosterone production during the early stages of testis development in order to try and reproduce the effects of impaired androgen action in the human testis. The present studies have not identified the age at which the seminiferous cords form in the marmoset, although it is clearly before 11 weeks gestation. The present study has shown the potential of the marmoset as a model for human testis development and therefore future studies to investigate cord formation in the fetal marmoset testis might allow the marmoset to be used as a complementary model for the above studies.

A second approach to investigating the critical events during human testis development and the impact of hormones and toxicants on the process would be to dissociate the cells of the human fetal testes prior to xenografting. The dissociated

cells could be genetically manipulated or treated with factors prior to grafting. It has been demonstrated that dissociated fetal rat testis cells can reform testis cords after subcutaneous injection in nude mouse hosts (Honaramooz et al., 2007), (Kita et al., 2007). One intriguing possibility would be to mix isolated testicular cells from different stages of development prior to xenografting. This could be used to test the hypothesis that predisposition to CIS may be associated with asynchronous differentiation or delayed maturation of germ (or Sertoli) cells. Use of the marmoset for such studies would allow gonocytes from a fetal testis to be mixed with the cells from a juvenile animal to develop a model of delayed germ cell maturation in xenografts of these cell mixtures. This would provide a system in which the gonocytes would be exposed to a local environment with Sertoli cells and germ cells that are more advanced in their differentiation. A third approach to seminiferous cord disruption could be to introduce transgenes into the testis tissue. Such an approach has been used in bovine testis xenografts to introduce the β-galactosidase gene (Oatley et al., 2004). This technique could be used to introduce genes that either promote or disrupt normal cord formation and testis development.

The present studies have demonstrated that testis xenografting can recapitulate normal germ cell differentiation and proliferation using an in vivo system. This includes the use of early postnatal and juvenile marmoset testis. This means that xenografting may have wider implications than those already discussed in this thesis. One such potential use of xenografting juvenile tissue is in the study of fertility preservation in survivors of childhood cancer. These patients are at risk of infertility as a result of their treatment with cytotoxic agents. Currently, the only option available for fertility preservation in young males treated for cancer is semen cryopreservation. For pre-pubertal patients, techniques for fertility preservation remain theoretical and as yet unproven. Xenografting of testis material is one potential technique, which would involve removal of tissue prior to treatment. This material could be xenografted in order that the germ cells differentiate. Mature

germ cells could be retrieved for use in assisted reproductive technologies (Jahnukainen et al., 2006). In the present studies juvenile marmoset xenografts survived and developed as xenografts. Germ cells within these grafts are preserved and tubular development including lumen formation occurs. The demonstration of the similarity between testis development in the human and marmoset suggests that juvenile xenografts may be used in studies relating to fertility preservation in children. Differentiation of germ cells from immature testis tissue has been shown to be possible for several species including mice and pigs in which sperm can be produced in xenografts that are capable of fertilising eggs using intracytoplasmic sperm injection (Schlatt et al., 2003), (Honaramooz et al., 2008). This can result in the production of progeny from xenograft derived sperm (Schlatt et al., 2003). Transplantation of immature testis tissue from 13 month old rhesus monkeys, subcutaneously onto the back of ICR/SCID mice resulted in accelerated spermatogenesis with production of mature sperm. However as discussed previously the rhesus monkey has some differences in the organisation of spermatogenesis compared to the marmoset and human (Millar et al., 2000); (Sharpe, 2003a) and as a result developing this system in the marmoset may be more relevant.

Previous studies by other groups have largely involved xenografting of newborn marmoset testes and have not been grafted for an equivalent length of time in which spermatogenesis would have been achieved in the intact *in situ* marmoset testis (Schlatt et al., 2002), (Wistuba et al., 2004), although autologous transplants of juvenile marmoset testis did initiate meiosis they failed to develop any further (Wistuba et al., 2006). Further studies of juvenile marmoset grafting are required using longer periods of grafting and manipulation of the local environment in order to establish whether this approach could be applied as a model of fertility preservation in prepubertal human patients undergoing cytotoxic treatment for conditions such as childhood cancer. This is the first study to have demonstrated

differentiation of human germ cells within testis xenografts and this is also of importance in the context of fertility preservation studies. Previous studies have shown that germ cells within the human fetal (Yu et al., 2006), (Skakkebaek et al., 1974) and juvenile (Goossens et al., 2008), (Wyns et al., 2008) testis can survive as xenografts, however these germ cells did not show development during the grafting period. Longer periods of grafting are required to determine whether human testis xenografts are capable of undergoing meiosis. The demonstration that human fetal testis grafts produce basal and inducible testosterone means that the hormonal environment can be controlled in these grafts to mimic that of the normal human testis. It has been shown that gonadotrophin treatment of mice receiving xenografts of immature rhesus monkey testis results in termination of the childhood period of development and acceleration of spermatogenesis (Rathi et al., 2008). This means that hormonal stimulation of immature human testis xenografts may be able to shorten the childhood period and allow the completion of spermatogenesis. The present studies have grafted human fetal testis for up to 5 months, with good preservation of the testis tissue. Longer periods of grafting including investigation of the effects of gonadotrophin stimulation are required to assess whether human testis xenografts can progress through meiosis.

In conclusion, this study has investigated normal and abnormal germ cell development in the human and marmoset testis. Studies in human patients with TGCT have characterised various protein expression profiles of differentiation and proliferation in CIS cells, which support their origin from fetal germ cells, in addition to identifying novel expression profiles. *In vivo* systems have been developed to investigate testis development in humans and marmosets during fetal and postnatal life. The marmoset has been shown to be a good model for human fetal and postnatal testis development. Xenografting of testis tissue from human and marmoset testes into nude mice hosts can recapitulate the normal development of the testes. This includes germ cell differentiation and proliferation in addition to

initial formation of the seminiferous cords. Use of the marmoset provides the opportunity to investigate human disorders of testis development by manipulation of normal testis development in the intact animal, or alternatively by use of the xenografting approach. Xenografting of human and marmoset testis tissue may also be used to study the development of DSD and CIS/TGCT, which have their origins in fetal life, or alternatively for studies relating to fertility preservation in children. Initial attempts to disrupt testis development with exposure to phthalates have not demonstrated any effects on primates, however further investigation using the systems developed in this thesis is required.

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